THE CULTURE AND IDENTIFICATION OF GRAM-NEGATIVE ANAEROBIC BACILLI OF CLINICAL INTEREST, WITH SPECIAL REFERENCE TO THE USE OF GAS CHROMATOGRAPHY

A.G. DEACON

B.Sc., M.Sc. (Otago).

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Plate I.

FRONTISPIECE

The Pye Unicam series 104 Gas Chromatograph used in these studies

PREFACE

Recent reviews reflect an increasing understanding of bacterial anaerobiosis with reference to the anaerobic requirement of some bacteria for growth and the varying oxygen sensitivities of different species or strains. These features underlie practical problems in the isolation and identification of these organisms. Recent improvements in anaerobic culture methods have solved some of the technical difficulties. Different groups of workers have disagreed on the type of approach required: some have shown the value of anaerobic cabinets or roll-tube systems that achieve the complete exclusion of oxygen during all handling procedures in the laboratory. The Edinburgh workers and others have however shown that such extreme approaches are unnecessary in the clinical laboratory. They have demonstrated that it is possible to recover quantitatively similar numbers of obligate anaerobes on solid media from clinical specimens with carefully controlled methods performed on the open bench, with subsequent incubation of media in anaerobic jars.

When the present author joined the Edinburgh team some growth failures were being experienced during the characterisation of clinical isolates from the family Bacteroidaceae. Accordingly these problems were investigated. It was recognised that growth failures can be caused by

a variety of factors of a technical or nutritional nature and these are examined in this thesis.

It was first necessary to re-examine some aspects of the cultural methods in use. Studies confirmed and extended the previous conclusions of the Edinburgh team and showed that quantitative levels of growth in liquid media are similar with either an anaerobic cabinet and pre-reduced media, or with the standardised anaerobic culture procedures in routine use. The importance of pre-steaming liquid media before inoculation at the bench was confirmed and the actual degree of reduction produced by this step was demonstrated quantitatively with an electrometric device. The device was also adapted to monitor the establishment and maintenance of anaerobic conditions in the anaerobic cabinet used in the direct comparisons performed. When it was clear that growth failures in characterisation media could not be attributed to the anaerobic methodology, a nutritional basis for the problem was sought.

In order to study the role of complex media requirements in this problem it was necessary to select a suitably demanding model; as most difficulty had been encountered with asaccharolytic strains of B. melaninogenicus it was decided to use a selection of strains of B. melaninogenicus ss. asaccharolyticus that seemed to show representative

degrees of difficulty. Observations on these strains were extended with tests on strains from other species or subspecies of the genus as necessary. A wide range of media and possible growth supplements were tested and the effect of minor variations in a successful medium were investigated. Effective media were developed for various purposes including the analysis of the fatty acid end-products of a range of clinical isolates by gas-liquid chromatography (GLC). Media were also evaluated for the performance of routine fermentation tests as part of the characterisation scheme used for identifying the isolates.

The resolution of the growth difficulties enabled the study of the effects that differences in media can have on the fatty acid end-products of fermentation of strains of Gram-negative anaerobic bacilli. The limitations of this approach to the identification of these organisms were evaluated and placed in perspective in relation to a wide range of morphological, biochemical, tolerance and antibiotic resistance tests used in the characterisation of clinical isolates from the family Bacteroidaceae. Variations were observed in the fatty acid profiles of single strains tested in more than one medium; there was culture to culture variation in the same media on different occasions; and variations were noted in fermentation test results of single strains tested

in different media.

Attempts to take account of major factors responsible for some of the variations, led to the development of a definitive GLC procedure for the analysis of the end-products of metabolism. It was also necessary to study the variations in fermentation test results in detail. Glucose utilisation experiments demonstrated that some asaccharolytic strains can utilise small amounts of glucose and made it possible to differentiate these strains from weakly asaccharolytic strains.

The established characterisation scheme for the Gram-negative anaerobic bacilli was then extended to include additional tests that allowed identification of strains from previously untested species and subspecies of clinical importance.

ABSTRACT OF THESIS

The literature on the classification and nomenclature of the family Bacteroidaceae and the occurrence on these organisms as commensals or pathogens in Man and other animals is reviewed.

The historical development and principles of modern methods of anaerobic culture are briefly reviewed and discussed in relation to current hypotheses on the nature of anaerobiosis and theories on the oxygen sensitivity or tolerance of clinically important bacteroides strains. Preliminary studies are presented on additional aspects of the cultural procedures previously developed by Edinburgh workers. The use of an electrometric device for the measurement of redox potentials in samples of a broth medium and for monitoring the establishment and maintainence of extremely reduced conditions in an anaerobic cabinet is described. Conventional bench and anaerobic cabinet approaches to the culture of the Gram-negative, non-sporing anaerobic bacilli are compared with further oxygen-sensitive strains not previously tested by Edinburgh workers and the earlier findings extended to show that quantitative recoveries of exacting strains in liquid media are similar with either approach.

The nutritional requirements of the Bacteroidaceae are reviewed and extensive growth studies in a range of complex media are described. Media based on ordinary bacteriological peptones are shown to be inadequate for the growth of strains of B. melaninogenicus ss. asaccharolyticus, but media containing peptide-rich Proteose peptones are shown to support good growth of the strains tested. A balanced salts solution described by other workers was found to inhibit the growth of strains of B. melaninogenicus ss. asaccharolyticus, and glucose was shown to cause an increased lag phase in the growth of the same strains. High concentrations of vitamin B_{12} (cyanocobalamin) stimulated the growth of strains of B. melaninogenicus ss. asaccharolyticus.

Methods of identifying the Gram-negative, non-sporing anaerobic bacilli are reviewed. The application of gas chromatographic (GLC) techniques to identification of bacteria and the role that these approaches have played in resolving the confused taxonomy of different bacterial groups are considered in detail. Techniques for the gas-liquid chromatographic separation and identification of short-chain fatty acid metabolic products of 185 isolates from the family Bacteroidaceae were separated and identified and a range of possible media evaluated for this work. The limitations of the GLC approach to the identification of a wide range of strains from various clinical sources

are determined and the results correlated with those of a series of morphological, biochemical, tolerance and antibiotic resistance tests. The potential application of GLC procedures to the rapid and accurate identification of these organisms in hospital laboratories is considered.

An established characterisation scheme for the identification of the Bacteroidaceae on the basis of conventional tests is enlarged by the inclusion of additional tests and the range of discrimination of the scheme is widened after studies with selected strains. Problems in fermentation testing are examined with selected strains of B. melaninogenicus and the detailed results of fermentation and glucose utilisation studies with these strains are presented.

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I am especially grateful to Mrs Vivien McGrath for her patience, care and skill in typing this manuscript. I am also indebted to the staff of the Medical Illustrations Service of the University for their help in the preparation of the illustrations in the thesis.

My wife Dale has been a constant source of love and understanding throughout my studies both here in Edinburgh and previously in New Zealand and it is a pleasure to record my gratitude to her. She has been particularly patient and tolerant during the gestation of this thesis at a time when she has been heavily committed both in her work and in looking after our newborn son, Russell and I wish to especially thank her for her care and support.

These studies have been supported by a grant from the Medical Research Council and I wish to record my appreciation for this assistance.

DEDICATION

This thesis is dedicated to the memory of my parents who gave me much love and encouragement.

LIST OF PAPERS DERIVED FROM THIS WORK

The results of some of the work presented in this thesis have already been published or are in press. The relevant references are:

(i) HOLBROOK, W.P., DUERDEN, B.I. AND DEACON, A.G. (1977). The classification of Bacteroides melaninogenicus and related species. J. appl. Bact., 42, 259-273.

(ii) DEACON, A.G., DUERDEN, B.I. AND HOLBROOK, W.P. (1978). Gas-liquid chromatographic analysis of metabolic products in the identification of Bacteroidaceae of clinical interest. Accepted for publication in J. Med. Microbiol. Likely date of publication February, 1978. Documentary evidence of acceptance is enclosed.

DECLARATION

The investigations and procedures that form the basis of this thesis were personally designed and performed or supervised by the author.

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SUMMARY

The literature on the classification and nomenclature of the family Bacteroidaceae is reviewed with emphasis on recent changes and listing the currently recognised, or proposed, species and subspecies of the three major genera of clinical importance. The occurrence of the Bacteroidaceae as significant components of the normal commensal flora of man and other animals and the involvement of some species or subspecies in a wide variety of infections, often of endogenous origin, is reviewed.

The historical development of anaerobic culture techniques and the principles of modern methods are briefly reviewed and discussed in relation to current hypotheses on the nature of anaerobiosis and the possible factors involved in the oxygen sensitivity or tolerance of clinically important bacteroides strains.

Preliminary studies are presented on additional aspects of the cultural procedures previously established by Edinburgh workers. An electrometric device was constructed and used to measure the redox potentials in containers of locally prepared and aerobically stored Robertson's cooked meat broth. Evidence is presented that the reducing substances present in the meat particles are unable to produce and maintain very reduced conditions either in the broth above the meat or in the depths of the meat layers.

The findings are not consistent with the previous observations of a much-quoted study on this medium, and possible reasons for the discrepancy are discussed. Quantitative evidence is presented to demonstrate the effectiveness of pre-steaming liquid media prior to inoculation to accomplish an initial reduction by removal of dissolved oxygen. The importance of this step as part of a standardised, open-bench, anaerobic culture procedure was re-affirmed.

The adaptation of the platinum electrode system to monitor the establishment and maintenance of extremely reduced conditions in an anaerobic cabinet is described. By monitoring the Eh levels produced in an uninoculated organic broth medium, being continuously flushed with the exhaust gases taken directly from the cabinet, the device provided an effective and inexpensive alternative to commercially available oxygen analysers. Evidence is presented that the potentiometric system responded promptly and sensitively to brief contamination of the flushing gas with air. The evidence of other workers who used this approach is reviewed; further experiments are required to determine more precisely the rate and magnitude of the electrode response to defined amounts of oxygen under the conditions of test.

A re-appraisal of open bench and anaerobic cabinet approaches to the culture of obligate anaerobes of clinical

importance is described. The standardised anaerobic jar procedures used by the Edinburgh team were compared with an anaerobic cabinet approach, utilising pre-reduced and anaerobically sterilised media, for the recovery and subculture from small inocula of oxygen-sensitive strains representing a number of obligately anaerobic genera. Some of the strains belonged to species or genera not previously examined by members of the team. Comparable recoveries of the exacting strains tested were obtained by the anaerobic cabinet and the conventional anaerobic jar procedures either with solid media or with liquid media and the quantitative levels of growth were similar with both techniques. These findings confirm and extend the previous observations of the team.

The nutritional requirements of the Bacteroidaceae are reviewed and the significantly different requirements of the various species and subspecies within the group are noted. Problems of growth in characterisation test media encountered mainly with some strains of B. melaninogenicus are examined in detail and extensive growth studies with a range of complex media are reported with strains of B. melaninogenicus ss. asaccharolyticus as a model. Methods are described for comparing the growth obtained in test media by either spectrophotometric measurements or visual assessments of culture turbidity. The correlation of turbidity readings and visual assessments with total

cell counts is reported for single strains representing the species B. fragilis, the B. melaninogenicus subspecies asaccharolyticus and ss. intermedius, and also Fusobacterium polymorphum.

Less enriched media containing ordinary bacteriological peptones and either yeast or beef extracts were shown to be inadequate, failing to support good growth of the test strains of B. melaninogenicus ss. asaccharolyticus. A wide selection of possible growth supplements were unable to improve the growth of the test strains in a typical medium of this type.

Good growth of the test strains and of a wide range of clinical isolates of B. melaninogenicus was obtained in media containing the peptide-rich Proteose peptones. Combinations of Proteose peptones including Trypticase (BBL) and another Proteose peptone were shown to support most rapid growth rates to high population levels. Media containing fresh meat particles or horse serum were also shown to produce similar high growth rates. Strain-to-strain differences were noted in the growth rates of strains from the same subspecies in identical media grown under identical conditions. Glucose was found to cause a prolonged lag phase with strains of B. melaninogenicus ss. asaccharolyticus when it was added to media that were otherwise nutritionally adequate.

Glucose was also shown to stimulate the growth of saccharolytic strains of B. melaninogenicus from the subspecies melaninogenicus and ss. intermedius in Proteose peptone-enriched media. A balanced salts solution described by other workers was found to be inhibitory for strains of B. melaninogenicus ss. asaccharolyticus, but the growth of glucose-fermenting strains of B. melaninogenicus was unaffected. These observations are discussed in relation to the findings of other workers. Comparative growth studies, with unsupplemented Proteose peptone-based media and media supplemented with graded amounts of vitamin B_{12}, showed that strains of B. melaninogenicus ss. intermedius and ss. melaninogenicus, B. fragilis, B. thetaiotaomicron and B. vulgatus were not stimulated by the vitamin, one strain of B. melaninogenicus ss. melaninogenicus (previously designated B. oralis) was actually inhibited by high concentrations of the vitamin, and a high concentration of vitamin B_{12} stimulated the growth of the three test strains of B. melaninogenicus ss. asaccharolyticus.

Growth curves obtained by plotting data for sequential samples from bulk cultures of one of these test strains held under extremely-reduced conditions showed that the vitamin affected the slope of the logarithmic phase of growth but did not reduce the lag phase. Similar results

were obtained in test-tube cultures incubated in anaerobic jars. Differences between the time scale of the batch cultures in anaerobic jars with those in the bulk culture experiment were attributed to the additional time taken for the contents of anaerobic jars to reach the optimum growth conditions of temperature and redox potential.

Fatty acid production during fermentation by the type strain of B. melaninogenicus ss. asaccharolyticus was significantly affected by addition of vitamin B_{12} to the test media. Evidence is presented that there was uptake and binding of the vitamin by the test strains. Varying concentrations of DL methionine were not able to produce a similar stimulatory effect on the growth of the test strains. Some variability was noted in the response of the B. melaninogenicus ss. asaccharolyticus strains to the vitamin when tested on different occasions. The mechanisms by which the vitamin stimulated growth were not elucidated and further study with a defined medium is required. The possible significance of the present findings in terms of a possible role for strains of B. melaninogenicus ss. asaccharolyticus in the malabsorption of vitamin B_{12} associated with a gastrointestinal blind loop syndrome is discussed.

Methods of identifying and characterising the Gram-

negative non-sporing anaerobic bacilli are reviewed. The application of gas chromatographic techniques to the identification and characterisation of bacteria and the role that these approaches have played in resolving the confused taxonomy of different bacterial groups are considered in detail. Techniques for the gas-liquid chromatographic separation and identification of the short-chain, fatty acid metabolic products of anaerobic fermentation in broth cultures are described.

The acid end-products of 185 isolates from the family Bacteroidaceae were separated and analysed by gas-liquid chromatography on broth cultures. A range of media were evaluated and definitive studies were performed in a fully supplemented complex medium. The limitations of this approach to the identification of a wide range of strains from various clinical sources were determined and the results were correlated with those of a series of morphological, biochemical, tolerance and antibiotic resistance tests.

All test strains were identified to generic level on the basis of simple microscopic and colonial observations and GLC analysis; additional tests were required to allow species or subspecies identification of most strains. Population differences were detected between some species or subspecies by quantitative analyses of fatty acids,

but individual strains could not always be separated because of overlapping ranges of distribution of acids that were common products of more than one species or subspecies. Small differences in minor products between different species or subspecies were variable and are not considered adequate for discrimination at these taxonomic levels without support from other observations.

The potential application of the GLC technique to the rapid and accurate identification of these organisms in hospital laboratories is considered.

The established characterisation scheme used to identify all of the isolates studied was enlarged by the inclusion of additional tests that allowed the characterisation of species and subspecies not previously examined by the Edinburgh workers. The additional tests are described and evaluated with fifteen strains selected to extend the range of the characterisation scheme. The whole range of tests thus included morphological observations, biochemical, tolerance and antibiotic resistance tests, and gas-liquid chromatographic analysis of the fatty acid end-products of metabolism.

Problems in the fermentation testing of obligate anaerobes were examined with selected strains of

B. melaninogenicus. The methods used and the criteria accepted as evidence of fermentation are described. Glucose utilisation studies were performed in conjunction with the fermentation tests on the selected strains. A method is presented for the direct determination of glucose levels in broth cultures. The experiments demonstrated that some asaccharolytic strains of B. melaninogenicus utilise small amounts of glucose and made it possible to differentiate these strains from weakly saccharolytic strains. Detailed results of growth and glucose fermentation by the test strains in various complex media are presented and the difficulties of interpreting the results are discussed with reference to strain characteristics, and variations in media, growth conditions, criteria of fermentation, the period of incubation and the possible production of mutants by the test strains. The difficulties of fermentation testing encountered in the characterisation of the Gram-negative, non-sporing, anaerobic bacilli are discussed with reference to the present findings and to those of others.

It is submitted that the identification and characterisation of the Gram-negative non-sporing anaerobic bacilli to species and subspecies level requires a wide range of currently available tests, some of which are difficult to poise satisfactorily; there is clearly a need to continue the development of other tests reflecting stable characteristics for use in clinical laboratories.

INTRODUCTION

The Gram-negative anaerobic bacteria of the family Bacteroidaceae (Pribram, 1933) are non-sporing, uniform or pleomorphic rods or coccobacilli. A few species are motile with peritrichous flagella. They have been isolated from the alimentary tract of man and other animals and from insects; they are also found in the mouth of man and some animals and in the genital tract of the human female. Some species are pathogenic (Holdeman and Moore, 1974). An account of the occurrence and potential pathogenicity of this important group of bacteria is given in a later section.

Classification

The classification of this group has been confused, and changes in terminology during re-classifications have led to difficulties in nomenclature (Aalbaek, 1973). Extensive accounts of the changes in classification have been given by: Sebald (1962), Spiers (1971), Aalbaek (1973) and Holdeman and Moore (1974). The names used in the present account will largely follow those adopted by Holdeman and Moore (1974) in the 8th edition of Bergey's Manual and proposed by them in the Anaerobe Laboratory Manual of the Virginia Polytechnic Institute and State University (VPI: Holdeman and Moore, 1973). Certain more recent changes and additions to the designated genera will be described.

There are three currently recognised genera in the family Bacteroidaceae: Bacteroides, Fusobacterium and

Leptotrichia (Holdeman and Moore, 1974). The genera Desulfovibrio, Butyrivibrio, Succinivibrio, Succinimonas, Lachnospira and Selenomonas are designated in Bergey's Manual as "genera of uncertain affiliation". Recently the family was emended by Moore, Johnson and Holdeman (1976) to include monotrichous or lophotrichous bacteria. They proposed that the genera of uncertain affiliation be accepted and further that a new genus Desulfomonas be included in the family.

Alternative names used by some French workers were not accepted by the International Committee on Nomenclature of Bacteria (Beerens, 1970) on the recommendation of the Taxonomic Sub-committee on Gram-negative Anaerobic Rods. The genus Bacteroides now includes organisms previously designated Ristella, Zuberella and Eggerthella (Prévot, 1938; Sebald, 1962 and Beerens, Castel and Fievez, 1962). Similarly the named genera Fusiformis, Fusocillus, Sphaerophorus and Sphaerocillus (Prévot, 1938) were re-classified into the single genus Fusobacterium. This genus does not however include the organisms previously designated F. fusiforme or F. plauti-vincenti which were found to differ biochemically from the rest of the genus and were placed in the separate genus Leptotrichia (Judicial Commission, 1954, Opinion 13).

Bacteroides

The type species B. fragilis (Veillon and Zuber) was first described by Castellani and Chalmers (1919) but these workers did not report on the Gram stain characteristics of

their isolates. Initially the group was poorly defined, mainly on the basis of morphological characteristics and included both Gram-positive and Gram-negative strains. Gram-positive strains were excluded on the recommendation of Weiss and Rettger (1937). Prior to this, in studies on the Gram-negative anaerobic bacilli present in human faeces, Eggerth and Gagnon (1933) had described three species that they named B. convexus, B. vulgatus and B. ovatus and they established the first biochemical key for the group. B. convexus was shown to be identical with B. fragilis by Werner (1969) and then in 1970 Holdeman and Moore grouped these species together and accorded them subspecies status on the basis of their overall similarities to the original description of the species B. fragilis. The strains designated B. thetaiotaomicron (Distaso, 1912) were also regarded by them as a subspecies of the same species. Thus in 1973 the following subspecies were confirmed: B. fragilis ss. fragilis, ss. thetaiotaomicron, ss. distasonis, ss. vulgatus and ss. ovatus (Moore and Holdeman, 1973).

Holdeman and Moore (1974) stated that of 326 strains tested that resembled the original description of B. convexus (Eggerth and Gagnon) the majority did not exactly fit the listed phenotypic reactions given in the literature for the species B. fragilis but there was a continuum of variants and detailed analyses disclosed clusters of strains.

Subsequently, Cato and Johnson (1976) published their findings of a high degree of diversity in the arrangement of the DNA nucleotides of five reference strains representing each of the subspecies; they therefore recommended the re-instatement of species status for each of the accepted subspecies named above.

Beerens et al. (1971) had found the same strains to be serologically distinct. Accordingly species status is given to these organisms in this thesis.

Loesche, Socransky and Gibbons (1964) isolated Gram-negative non-sporing anaerobic rods from the human mouth and on the basis of morphological and biochemical observations, including acid end-product analyses on glucose-enriched media, they suggested a new species termed B. oralis. They recognised 2 subspecies, B. oralis ss. oralis and B. oralis ss. elongatus. Holdeman and Moore (1972) subsequently re-classified the latter subspecies as B. ochraceus (Prévot) on the basis of this strain's resemblance to strains first described by Prévot and his colleagues. The terms B. oralis and B. ochraceus have been used in the present study.

The production of a specific black pigment by Bacteroides spp. when growing on blood-containing media has been regarded as highly significant and diagnostic of strains of B. melaninogenicus (Beerens, 1970; Duerden, 1975). Duerden (1975) stated that

it remained to be seen whether this feature remains the significant basis of identification or whether antibiograms, gas-liquid chromatography of the acid end-products of glucose fermentation, or some other group of biochemical tests will provide more valid criteria for the taxonomy of the Bacteroides group.

The currently accepted name for the bacteroides organisms producing black-pigmented colonies when cultured on blood agar or lysed blood agar is B. melaninogenicus (Roy and Kelly, 1939; Holdeman and Moore, 1974). Oliver and Wherry (1921) first isolated these organisms from the mouth, abdominal wounds and faeces of humans and termed them Bacterium melaninogenicum. They thought the pigment was melanin, but Schwabacher, Lucas and Rimington (1947) concluded that the pigment was actually a haemin derivative. Tracy (1969) considered that it was colloidal ferrous sulphide, a black precipitate produced in fluid media by various Bacteroides-like organisms. The confusion was removed by the studies of Duerden (1975) who found that the pigment was in fact a haemin derivative and was entirely different from the ferrous sulphide producted by H_2S-producing organisms in the presence of ferrous ions.

At present there are three recognised subspecies of B. melaninogenicus: B. melaninogenicus ss. intermedius, B. melaninogenicus ss. melaninogenicus and B. melaninogenicus

ss. asaccharolyticus (Moore and Holdeman, 1973; Holdeman and Moore, 1974). It has been pointed out that it is against the established rules of taxonomy to have both asaccharolytic (B. melaninogenicus ss. asaccharolyticus) and saccharolytic (ss. intermedius and ss. melaninogenicus) organisms in the same species (Werner, Pulverer and Reichertz, 1971). Other workers have also commented on the substantial biochemical differences (Harding, Sutter, Finegold and Bricknell, 1976; Holbrook, Duerden and Deacon, 1977), and structural, physiological and genetic differences (Williams et al., 1975; Shah et al., 1976) between the asaccharolytic strains and the other pigmented subspecies. After a cooperative study in which the Edinburgh team took part, the International Committee for Systematic Bacteriology (ICSB) Taxonomic Sub-committee on Anaerobic Rods concluded that B. melaninogenicus ss. asaccharolyticus should be placed in a separate species Bacteroides asaccharolyticus (submitted for publication). This author was privileged to take part in these studies. The Edinburgh team sent a report to the ICSB Taxonomic Sub-committee; the report and the paper by Holbrook, Duerden and Deacon (1977) are bound in with this thesis. For the purpose of the present thesis, the nomenclature of Holdeman and Moore (1974) is retained.

The species B. corrodens consists of obligately anaerobic Gram-negative non-sporing bacilli that cause pitting in agar media (Eiken, 1958). Jackson and Goodman (1972) separated

these strains from facultative organisms with similar morphology and agar-digesting capability. The latter strains were placed in a separate genus Eikenella with the same species name (Jackson and Goodman, 1972). The facultative organisms differ from the obligately anaerobic strains in their ability to decarboxylate lysine, their inability to decompose urea and their higher content of guanine plus cytosine in their DNA (Jackson et al., 1971). The principal habitat of the anaerobic B. corrodens is the human mouth.

Bryant et al. (1958) isolated strictly anaerobic non-motile, Gram-negative rods from the rumen of calves. Many of their strains characteristically produced large quantities of succinic acid from carbohydrates. Three species of this group were named: B. succinogenes Hungate (Hungate, 1947; Bryant and Doetsch, 1954); B. amylophilus Hamlin and Hungate (Hamlin and Hungate, 1956); and B. ruminicola Bryant et al. (1958). The last-named species was divided into 2 subspecies: B. ruminicola ss. ruminicola included 8 biotypes that differed in H_2S production, gelatin liquefaction and the fermentation of a range of carbohydrates; B. ruminicola ss. brevis included 3 biotypes that differed in H_2S production and the fermentation of carbohydrates. The type species of B. ruminicola is B. ruminicola ss. brevis GA 33. Holdeman and Moore (1972) tested these strains in their extensive characterisation scheme and found that strains of B. ruminicola

ss. brevis differed from the non-pigmented B. oralis strains isolated from the mouth and characterised by Loesche et al. (1964) only in the fermentation of the pentoses arabinose and xylose. The relationship of these organisms occupying such dissimilar ecological niches is uncertain at this time.

Holdeman and Moore have proposed the new species names B. bivius and B. disiens for two non-pigmented Gram negative anaerobic bacilli regularly isolated from clinical material. The strains, previously not identified beyond genus level, were originally named Bacteroides Group PS and Bacteroides Group I respectively (VPI Anaerobe newsletter, October, 1974 and February, 1976; ICSB minutes 1976, submitted for publication). They were clearly not related at species level to any of the previously recognised species. The same authors have recently proposed the new subspecies name B. melaninogenicus ss. levii for the B. melaninogenicus Lev Group 1 strains originally isolated by Lev (1958). The strains are described as weakly fermentative strains producing n-butyric acid and requiring vitamin K and haemin for growth (Lev, Keudell and Milford, 1971).

A further new species, B. splanchnicus n. sp. has been described by Werner, Rintelen and Kunstek-Santos (1975). These non-pigmented, n-butyric acid-producing strains were isolated from human faeces and appendix tissue. Serologically they were found to be distinct from morphologically similar

strains of B. fragilis, B. thetaiotaomicron, B. vulgatus and B. distasonis. Additional strains in this genus are listed in table 1 below.

In the present work, which is primarily concerned with the culture and detailed identification of organisms in the Bacteroides genus, it has been necessary to take account of problems in the classification of species in related genera.

Fusobacterium and Leptotrichia

The taxonomy of the genus Fusobacterium is confused. There are several examples of disputed species and changes in nomenclature during successive re-classifications have led to a confusing list of synonyms for many of the currently accepted species. The previously designated genera Fusiformis, Fusocillus, Sphaerophorus and Sphaerocillus (Prévot, 1938) have been re-classified into the genus Fusobacterium (Aalbaek, 1973; Holdeman and Moore, 1974).

The original description of the genus Fusobacterium (Knorr, 1922) included three morphologically similar species: F. plauti-vincenti, F. nucleatum and F. polymorphum, but no type species was designated. Roy and Kelly (1939) and Breed (1948) designated F. plauti-vincenti Knorr as the type species. Spaulding and Rettger (1937) had distinguished two main groups of fusiform organisms on the basis of morphological and biochemical characteristics. F. plauti-

vincenti Knorr corresponded to Group II and F. polymorphum to Group I. F. nucleatum appeared to be similar to F. polymorphum. Bøe (1941) considered that the organisms designated F. plauti-vincenti Knorr were similar to the previously described strains of Leptotrichia buccalis (Trevisan, 1879) and proposed that F. plauti-vincenti be re-classified into this genus. Hoffman (1957) created further confusion by designating F. fusiforme (Veillon and Zuber) Hoffman as the type species of the genus Fusobacterium and regarded F. plauti-vincenti as synonymous.

Jackins and Barker (1951) had studied the fermentative processes of the fusobacteria and concluded that the differences between F. nucleatum Knorr and F. plauti-vincenti Knorr were substantial and suggested that they should not be placed in the same genus.

Baird-Parker (1960) and Omata and Braunberg (1960) confirmed the earlier findings of Bøe (1941) and Jackins and Barker (1951) and also proposed the re-classification of the species F. plauti-vincenti and F. fusiforme (Veillon and Zuber) Hoffman into the previously designated group L. buccalis. Holdeman and Moore (1974) pointed out that this name takes precedence and as the type species of the genus Fusobacterium was not valid the generic name has no nomenclatural standing. Pending a Judicial Commission Opinion they suggested that F. nucleatum should be designated as the new type species

of the genus Fusobacterium Knorr. Baird-Parker (1960) had recognised morphological differences between strains of F. polymorphum and F. nucleatum and despite the biochemical and fermentative similarities noted also by Spaulding and Rettger (1937), Bøe (1941) and Jackins and Barker (1951) he recommended that the two species names should be retained. Omata and Braunberg (1960) placed more stress on the metabolic similarities and agreed with the suggestions of Bøe and Jackins and Barker that these species should be regarded as synonymous. Holdeman and Moore (1974) confirmed their findings and also cited similarities in genetic composition to support this view. Pending a Judicial Commission Opinion both names are valid and in the present studies the species name F. polymorphum is used.

Hardie (1974) noted that at present Fusobacterium sp. from the human mouth are usually identified as F. nucleatum or F. polymorphum, but few investigators had studied them in detail. Holdeman and Moore (1974) retained all the oral fusobacteria in one species F. nucleatum and retained the genus Fusobacterium in the family Bacteroidaceae. In a recent paper, Page and Krywolap (1976) reported DNA base ratio and DNA-DNA hybridisation studies on single strains of F. polymorphum, F. fusiforme (sic) and Leptotrichia buccalis held in the American Type Culture Collection. The two Fusobacterium strains showed 78% homology whereas the

homology of these strains with the strain of L. buccalis was only 30%. The guanine plus cytosine (G + C) values of the strains were all similar and significantly lower than the values reported for other members of the family Bacteroidaceae. The authors consider the possibility of a close phylogenetic relationship between the genera Fusobacterium and Leptotrichia but suggest that the placement of all oral fusobacteria into one species and the placement of them within the family Bacteroidaceae requires re-evaluation.

Leptotrichia

The type strain of this species is L. buccalis Trevisan and the later synonyms F. fusiforme and F. plauti-vincenti (Hoffman, 1957) are invalid.

The majority of the names listed in the genus Fusobacterium in table 1 are new combinations attributed to Holdeman and Moore in several editions of the VPI manual (1970, 1972 and 1973). The authorities for the new combinations were confirmed by Moore and Holdeman (1973). Some previously described species have been deleted from the current descriptions in the 8th edition of Bergey's Manual either because no strains were available for re-testing or because the original descriptions were so incomplete that the strains cannot now be recognised.

Table 1: The currently recognised species and subspecies in the three principal genera of clinical importance in the family Bacteroidaceae

Family	:	BACTEROIDACEAE	
Genus	:	Bacteroides	Fusobacterium	Leptotrichia
Species and subspecies	:	B. fragilis (B. fragilis ss. fragilis)	F. nucleatum, Knorr	L. buccalis
		B. thetaiotaomicron	F. polymorphum, Knorr (valid synonym)	
		B. vulgatus	F. necrophorum	
		B. distasonis	F. necrogenes	
		B. ovatus	F. varium	
		B. splanchnicus (nov. sp.)*	F. symbiosum	
		B. disiens (nov. sp.)	F. gonidiaformans	
		B. bivius (nov. sp.)	F. naviforme	
		B. oralis	F. glutinosum	
		B. ochraceus	F. aquatile (previously F. novum)	
		B. melaninogenicus ss. intermedius	F. stabile	
		ss. melaninogenicus	F. mortiferum (F. ridiculosum)	
		ss. levii (nov. ssp.)	F. prausnitzii	
		ss. asaccharolyticus (B. asaccharolyticus)		
		B. corrodens		

Table 1: CONTD.

Family	:	BACTEROIDACEAE	
Genus	:	Bacteroides	Fusobacterium
Species and subspecies	:	B. ruminicola ss. brevis	F. plauti
		ss. ruminicola	F. bullosum
		B. succinogenes	F. russii
		B. amylophilus	
		B. multiacidus (nov. sp.)	
		B. eggerthii (nov. sp.)	
		B. hypermegas	
		B. serpens	
		B. termiditis	
		B. biacutus	
		B. constellatus	
		B. putredinis	
		B. pneumosintes	
		B. coagulans	
		B. praeacutus	

Table 1: CONTD.

Family	:	BACTEROIDACEAE
Genus	:	Bacteroides
Species and subspecies	:	B. nodosus
		B. furiosus
		B. capillosus
		B. niger

* All species labelled nov. sp. do not appear in the 8th edition of Bergey's Manual (Holdeman and Moore, 1974). See Holdeman and Moore (1974).

Identification of the Bacteroidaceae

The identification of Gram-negative anaerobic bacilli has presented problems partly because of the confusion in the classification of these bacteria. Different groups of workers have developed different classification systems with different names being given to the same organism and the same name given to obviously different organisms (Moore and Holdeman, 1973; Aalbaek, 1973). Recent co-operative efforts by members of the ICSB Taxonomic Subcommittee and other interested workers have encouraged the adoption of a uniform nomenclature in an effort to resolve some of the confusion (Holdeman, Cato and Moore, 1974). Further problems in identification of these organisms have arisen from the diversity of methods used. Duerden et al. (1976) stated that it was difficult to correlate the results of these different approaches and to establish the taxonomic level at which each operated.

The earliest observations on this group of organisms were based solely on morphological criteria and these features have continued to be a part of the often detailed and extensive characterisation schemes used by many present day workers. They have been criticised by various workers as unreliable or unhelpful (Beerens, 1952; Guillaume, Beerens and Osteux, 1956; Sebald, 1962; Tannock, 1977). Other workers consider that such observations are valuable in establishing the genus of an isolate (Cato et al., 1970; Moore and Holdeman, 1972).

Many phenotypic characteristics have been used to identify the Bacteroidaceae. The fermentative processes of the fusobacteria were studied by Jackins and Barker (1951) and by Loesche and Gibbons (1968). Biochemical reactions have supplemented morphological observations in the studies by numerous workers on fusobacteria; (Hine and Berry, 1937; Spaulding and Rettger, 1937; Omata and Braunberg, 1960) and on Bacteroides spp. (Sawyer, Macdonald and Gibbons, 1962; Prévot, 1966; James and Robinson, 1975; Williams et al., 1974 and 1975; Harding, Sutter and Finegold, 1975). Numerical taxonomic techniques, utilising a wide range of tests, have been used by Barnes and Goldberg (1968) and by Sundqvist (1976).

A few useful laboratory manuals have been published in recent years to assist clinical and research microbiologists in identifying these isolates. Detailed descriptions of media and methods accompany the results of extensive morphological observations, biochemical tests and gas chromatographic analyses performed on many recognised species and subspecies of anaerobes (Cato et al., 1970; Holdeman and Moore, 1972; Sutter et al., 1972; Sutter, Vargo and Finegold, 1975). The increasing need in clinical laboratories for a relatively simple characterisation scheme to identify clinically important members of the family Bacteroidaceae to species or subspecies level without recourse to sophisticated tests of metabolic or genetic relatedness was recognised by

Duerden et al. (1976). These workers developed a wide range of simple, carefully controlled tests able to be used in conjunction with conventional anaerobic jar procedures (Collee et al., 1972). A combination of morphological observations, biochemical tests, antibiotic resistance, dye and bile salt tolerance tests were used to identify 165 reference and laboratory isolates of Gram-negative anaerobic bacilli. A scheme was presented for the identification of unknown laboratory isolates (see also Holbrook, 1976).

At a less differentiated taxonomic level, variations in antibiotic resistance patterns have been used by Finegold, Harada and Miller (1967); Sutter and Finegold (1971); Werner (1972) and Peach (1975). Similarly dye tolerance tests were employed by Suzuki, Ushijima and Ichinose (1966) and bile salt sensitivity patterns were studied by Shimada, Sutter and Finegold (1970).

Prévot (1966) considered that fatty acid end-product analyses were of value in determining the genus of isolates. He noted however that generic definitions at this level on the basis of these characteristics could only be formulated on the basis of positive findings and not on the absence of a particular end product. Thus, for example, the generic name Desuccinivibrio was considered invalid (Prévot, 1966). Cato et al. (1970); Moore (1970) and Holdeman and Moore (1974) have further stated that most anaerobes can be assigned

to a defined genus on the basis of morphological characteristics, including the Gram reaction, and on the metabolic products of fermentation in complex media. These characteristics form the basis of the currently recognised classification for the family Bacteroidaceae (Holdeman and Moore, 1974).

The analysis of metabolic end-products in broth cultures of Bacteroides spp. was first described by Guillaume, Beerens and Osteux (1956) using distillation and paper chromatographic methods of separation and identification. More recently, gas chromatographic techniques have replaced the earlier time-consuming and laborious procedures. The role of gas chromatography in resolving some of the problems in bacterial taxonomy and its possible role in identifying clinically important anaerobic species is considered in more detail below.

Other approaches have included the early diagnosis of infections involving B. melaninogenicus strains on the basis of a brick red or pink fluorescence produced when swabs of young, as yet non-pigmented cultures are placed under a Wood's lamp (Myers et al., 1969).

Funderburk and Kester (1975) used differences in the electrophoretic mobility of valine, malic and pyruvic dehydrogenases to differentiate strains of Bacteroides. Cell wall analyses have been used by Williams et al. (1974,

1975) and by Shah *et al.* (1976) and isolates from the genera *Leptotrichia* and *Fusobacterium* have been distinguished by differences in their cellular proteins as determined by gel electrophoresis (Baboolal (1972). Strom *et al.* (1976) also used polyacrylamide gel electrophoresis to characterise and identify the protein profiles of nine strains from the genus *Bacteroides* and two strains from the genus *Fusobacterium* representing a total of six species. The profiles were considered sufficiently distinct and reproducible to allow identification at subspecies level or even the detection of minor strain differences.

Serological tests have been used by Beerens *et al.* (1971); Sharpe (1971) and Lambe (1974). Bacteriocin production has been studied as a possible aid to identification by Podhaisky and Reinhold (1970) and by Collee, Watt and Dewhurst (1974). Genetic differences in the group have been investigated by various workers (Sebald, 1962; Johnson, 1973; Williams *et al.*, 1974 and 1975; Cato and Johnson, 1976; Shah *et al.*, 1976; and Page and Krywolap, 1976). The studies of Shah *et al.* (1976) illustrate another up-to-date approach to resolving some of the problems in the classification of *B. melaninogenicus* strains. These workers examined the biochemical properties of 45 *B. melaninogenicus* strains, representing the three currently recognised subspecies, with a combination of simple morphological and physiological observations and a number of sophisticated tests including

gas chromatography of the fatty acid end-products, analyses of the basic amino acid content of the cell wall mucopeptide, base composition of the DNA, the electrophoretic mobility of malate dehydrogenase and the susceptibility to selected antibiotics.

Problems in Bacterial Taxonomy and the role of gas chromatography

Problems of bacterial taxonomy for convenience may be considered in general and specific categories. The general problems of large numbers of isolates, the lack of morphological complexity, the lack of a fossil record and the lack of a suitable definition of a species at first do not seem of direct concern to the microbiologist faced with a specific problem of what to label an isolate which does not appear to fall into any of the previously described groups. However, in dealing with specific problems it soon becomes apparent that the microbiologist must concern himself with the broader aspects (as they are the very basis and cause of his problems). Continuing improvements in pre-existing cultural techniques and the development of new approaches have contributed to the general problems in that large numbers of previously unrecognized bacterial strains are being isolated and detailed examination of these strains is highlighting inadequacies in earlier descriptions and

taxonomic groupings. Inadequate descriptions based on colonial and microscopic appearances and a limited range of biochemical and serological tests have made identification of many of these isolates difficult either because they belong to species not previously described or because they belong to species so incompletely described that they cannot now be recognized (Moore, Cato and Holdeman, 1969). In recent years increasing use has been made of a wider range of biochemical and serological tests and the more sophisticated or technically difficult tests of structural, genetic and metabolic relatedness in conjunction with computer analysis and retrieval of information to assist in the resolution of the problems and areas of confusion in the earlier taxonomic schemes. One of the most useful and versatile of these approaches has been the implementation of gas chromatographic techniques by microbiologists. The feasibility of its use in classification of micro-organisms was first reported by Abel, DeSchmertzing and Peterson (1963) and by Brown and Cosenya (1964) and Yamakawa and Ueta (1964) and since that time gas liquid chromatography (GLC) has been used in an ever widening range of applications to assist in the identification and classification of micro-organisms. It is worthwhile to review this range of applications and to get the role of GLC into perspective with reference to the characterisation of bacteria; and it is important to give special consideration to the practical aspects of its

clinical potential and the present usefulness of GLC in microbiology.

The first realisation that chromatographic techniques could be developed for the separation and identification of chemical mixtures has been attributed to Tswett (1903) by Mitruka (1975) who reviewed the historical development of chromatographic techniques for these purposes. Martin and Synge (1941) introduced column partition chromatography, developed paper chromatography, discovered liquid-liquid partition chromatography and suggested the use of an inert gas as a mobile phase in a column separation. The suggestion was taken up by James and Martin (1952) who first demonstrated the separation of steam volatile fatty acids by gas-liquid chromatography and used a titration method for the quantitation of the separated compounds. Griffith, James and Phillips (1952) developed the first thermal conductivity katharometer detector system for use in gas chromatography.

At the present stage of its development as an analytical tool, gas-liquid chromatography (GLC) permits the rapid and sensitive separation and analysis of the volatile components of complex organic mixtures. In additon, many non-volatile compounds present in biological material may be readily converted to volatile derivatives, thus widening the scope of the analysis even further. The variety of gas chromatographic techniques and the wide range of column packings available

at present permit the differentiation of entire classes of compounds within a few minutes (Cherry and Moss, 1969). In microbiological research two basic approaches have been developed: i) the analysis of pyrolysis products of bacterial cells, and ii) the analysis of extracted cells, growth medium or headspace gas without pyrolysis.

Pyrolysis, followed by GLC, originally suggested as a means of detecting microbial life on the planet Mars by Oyama (1963), involves the controlled high temperature decomposition of cellular material in an inert atmosphere. The volatile pyrolysis products can be separated on appropriate columns and analysed with a suitably sensitive detector. Three types of pyrolysis reactions have been developed: the Curie Point method, the heated filament, and the tube furnace. Of these, the Curie Point method is preferred for its accuracy and reproducibility (Andrews, 1970). Reiner (1965) gave an early indication of the possible taxonomic use of pyrolysis - gas liquid chromatography (P-GLC) when he attempted the differentiation of enterobacteria on the basis of fatty acid pyrolysis products of whole cells. Reiner and his co-workers continued to develop this approach (Reiner, 1967; Reiner and Ewing, 1968) and extended it to taxonomic studies of the mycobacteria (Reiner and Kubica, 1969). More recently, P-GLC was applied to the differentiation of Clostridium botulinum types A, B and E by Cone and Lechowich (1970); these workers regarded the procedure as a promising

analytical tool for identification of C. botulinum at the type level, but they stressed the need for further investigation and testing of the operating parameters before the technique could be applied at the clinical level for detecting C. botulinum in food products. Cherry and Moss (1969) had meanwhile drawn attention to the problems associated with standardisation of the pyrolysis step and noted that this factor in conjunction with the other variables of composition of media, conditions of growth and age of the cells, resulted in a lack of reproducibility of results between laboratories. Other difficulties noted by these authors were the problems of identifying the individual peaks representing the various substances in the chromatographic profiles and the requirement for computer analysis of this data. Despite these undoubtedly major drawbacks, Cherry and Moss considered that P-GLC was likely to play an important role in the analysis of biological constituents of high molecular weight found in bacteria.

The possibility that a non-pyrolytic approach to the gas-chromatographic analysis of the chemical composition of bacteria might offer a reliable and less time-consuming aid to clarifying the taxonomic position of some bacterial groups was first explored by Abel, DeSchmertzing and Peterson (1963) and shortly thereafter by Brown and Cosenya (1964) and by Yamakawa and Ueta (1965). Of particular interest to these workers were the medium- and long-chain fatty acids

(C_9-C_{20}) of the cell wall that could be directly saponified, extracted and converted to volatile methyl esters suitable for gas chromatography. Yamakawa and Ueta (1965) also extracted and esterified cell wall monosaccharides of Neisseria spp. for the same purposes. These techniques, or modifications of them, have been subsequently applied to the study of both whole cell and discrete fraction hydrolysates of the cellular fatty acids or phospholipids in a wide range of bacterial groups including the clostridia (Moss and Lewis, 1967; Farshtchi and Moss, 1969, 1970), propionibacteria (Moss, Dowell Jr., Farshtchi, Raines and Cherry, 1969), bifidobacteria and lactobacilli (Veerkamp, 1971), Escherichia coli (Weinbaum and Panos, 1966) and Bacillus sp. (Kaneda, 1967, 1968).

In the years following the demonstration by Abel et al. (1963) of the role that lipids might play in leading to a better classification of bacteria there has been a rapid expansion of knowledge relating to bacterial lipids. An increased awareness of their importance in cellular processes and improved techniques of study are thought to have contributed to this expansion (Shaw, 1974). A number of extensive reviews of this information have appeared (Kates, 1964; Lennarz, 1970; Shaw, 1970). In his most recent review, Shaw (1974) reassessed the literature in an attempt to determine the value of lipid composition as a guide to classification. Shaw defined 3 criteria that he considered

should be used to assess any chemotaxonomic method:

i) "It must be applicable to as large a number of organisms as possible;

ii) the required information should be readily obtained; and

iii) the parameters utilised should differ as widely as possible from one genus or family to the next."

Shaw (1974) concluded that lipid composition studies go further than any other chemotaxonomic method towards satisfying these criteria. Other workers have investigated another approach with some success: the separation and analysis of the metabolic products of bacteria.

The use of GLC for analysing metabolic products and the use of such data in classification and identification in fact developed concurrently with GLC studies on the chemical composition of bacterial cells. The development of both approaches has subsequently led to the accumulation of a large amount of information and has helped to clarify taxonomic relationships among micro-organisms (Cherry and Moss, 1969). The metabolic products of particular interest for taxonomic purposes have been the steam volatile short chain (C_2-C_6) fatty acids produced by bacterial fermentations in organic media.

Knowledge of bacterial metabolism has developed continuously since the early days of bacteriology and it has been well established that many different groups of bacteria are able to produce a vast array of products during their fermentation of proteinaceous or carbohydrate-containing media. It is generally recognised that the fermentation products obtained vary with different species and that the range and amounts of the products produced by a particular species or strain are dependent on a variety of factors. These include the organism's growth phase at the time of its inoculation into the test medium, the composition and pH of the medium, and the prevailing cultural conditions (Lewis, Moss and Jones, 1967). Stephenson (1949) noted that the mode of attack on carbohydrates was more dependent on these factors than on the nature of the substrate.

The pathways employed for fermentation of carbohydrates such as glucose vary widely in different micro-organisms. Isotope-labelling studies have shown three main pathways of glycolysis that may occur (Wood, 1961). The most common is the Embden-Meyerhof pathway. Glucose initially undergoes phosphorylation to glucose-6-phosphate and glycolysis proceeds via a series of intermediates to pyruvic acid. Both the Entner-Doudoroff and hexose monophosphate pathways offer alternative glycolytic routes to pyruvic acid. By whatever pathways carbohydrate is broken down however, the

key metabolite has been shown to be pyruvic acid. The subsequent fate of pyruvic acid is determined by the conditions and enzymic capabilities of the organism: many bacteria have the enzymic ability to produce a wide range of products from pyruvic acid including acetic, formic, lactic, succinic, butyric and fumaric acids, ethyl and isopropyl alcohol, acetone, glycerol, acetylmethyl carbinol, butylene glycol, carbon dioxide and hydrogen. A summary of the important reactions giving rise to these products may be found in Topley and Wilson's Principles of Bacteriology and Immunity (Ed. Wilson and Miles, 1964). More extensive accounts include those of Wood (1961) and Stanier, Doudoroff and Adelberg (1971).

The breakdown of amino acids may occur as a result of deamination, decarboxylation or transamination. Bacterial deamination may take place by four direct pathways and one indirect path, the Stickland reaction (Stickland, 1934). The range of products resulting from deamination may include ammonia, a keto acid, a saturated or unsaturated fatty acid and a hydroxy acid. In some anaerobic bacteria, particularly proteolytic strains unable to utilise carbohydrate, the significant energy-yielding process is thought to be the Stickland reaction. It is a coupled oxido-reductive deamination involving two amino acids resulting in the production of a keto acid and a saturated fatty acid (Nisman, 1954; Barker, 1961; Drasar and Hill, 1974). The up-to-date review of the bacterial metabolism of nitrogen compounds

by Drasar and Hill (1974) provides a useful summary of these microbial degradations.

GLC methods for the analysis of short-chain (C_2-C_{10}) volatile fatty acids had been used in various research and industrial applications for some years before their first use of microbiologists. The use of GLC for these analyses, originally developed by James and Martin (1952; 1956), was an improvement on the silica gel-liquid partition chromatography columns already in use (Elsden, 1946). By 1965 Kaplanova and Janak (1966) noted that the system of analysis was well worked out and widespread in its application. Considerable refinement of the instruments, and in particular of their detection systems, had been achieved. It was at this time that the first reports of the microbiological applications were seen. Bassette and Claydon (1965) used a headspace vapour sampling technique to analyse the products of bacterial fermentation in milk and Packett and McCune (1965) reported the direct GLC analysis of free aqueous volatile fatty acids produced by bacterial fermentation in culture media. The first detailed investigations of this approach to taxonomy were undertaken by Moore, Cato and Holdeman (1966) and by Henis, Gould and Alexander (1966). Moore and his colleagues separated and identified the fatty acids and alcohols produced by representative strains from each of 20 species of the genus Clostridium. They utilised a thermal conductivity detector system for the GLC analyses

and the results were compared with results obtained from the same samples after chromatography with a silicic acid liquid chromatography column. Moore and Cato (1965) had previously used the latter method and found that all clostridial species and many non-sporing obligate anaerobes produced well defined and reproducible fermentation patterns. The 20 species of clostridia were separated into 8 fermentation groups by GLC and further subdivision of some groups was possible with the liquid chromatography procedures (Moore et al., 1966). Henis, Gould and Alexander (1966) used a more sensitive flame ionisation and electron capture detection system for the analysis of fatty acids, alcohols, acetoin and 2, 3 butanediol produced by 6 species of Bacillus and individual strains of E. coli, K. aerogenes and P. aeruginosa. Different GLC profiles or signatures were obtained from the various genera, species and even from strains of the same species.

Analyses of the fatty acid and alcohol end-products of fermentation in peptone-based media have played a significant role in simplifying the identification and classification of obligately anaerobic bacteria. In the 8th edition of Bergey's Manual (Holdeman and Moore, 1974), descriptions of the genera in the family Bacteroidaceae are based mainly on the fatty acid end-products in PY and PYG media (Holdeman and Moore, 1972). Thus the principal genera are described as follows:

"Genus I Bacteroides; produce (from peptone or glucose) mixtures of acids including succinic, acetic, formic, lactic, propionic; butyric acid usually not a major product. Some species produce a mixture of butyric, iso-butyric and iso-valeric acids, along with major amounts of succinic acid.

Genus II Fusobacterium; produce (from peptone or glucose) butyric acid (without iso-butyric or iso-valeric acids) as a major product.

Genus III Leptotrichia; produce (from peptone or glucose) lactic acid as the only major fermentation acid."

Gas chromatographic analyses of the fatty acid fermentation products of organisms from the family Bacteroidaceae were first reported by Werner (1969) and Cato et al. (1970). Subsequently, numerous workers have utilised this approach as an aid to the identification of Gram-negative anaerobic bacilli isolated from various human and animal sources. A variety of methods have been employed for the separation and detection of the fatty acids. The VPI Anaerobe laboratory methods (Moore, 1966; Cato et al., 1970; Holdeman and Moore, 1972) was based on the extraction of volatile acids in diethyl ether, their separation on a suitable column and detection in a thermal conductivity detector (TCD). The non-volatile acids were esterified with methanol and sulphuric acid; the esters extracted with chloroform and separated on the same column used for the volatile acids. Improved columns

have been subsequently used and recommended by Moore and his colleagues (Hauser and Zabransky, 1975).

Rogosa and Love (1968) had shown that volatile fatty acids in fermentation media could be separated directly on a porous polymer column packing and identified with a flame ionisation detector (FID). Sutter et al. (1972 and 1975), Bricknell and Finegold (1973) and Bricknell, Sutter and Finegold (1975) recommended direct analyses of acidified cultures with an FID. For non-volatile analyses, they recommended a boron-trifluoride methanol mixture for esterification followed by chloroform extraction and analysis by the procedures of Holdeman and Moore (1972). Carlsson (1973) adopted a similar approach for the analysis of the volatile fatty acids. Prior to analysis the culture samples were passed through a cation exchange resin to remove non-volatile impurities. The procedures of Carlsson were used in the studies reported in this thesis with only slight modifications (see the Materials and Methods section).

Two recent papers have described gas chromatographic methods for the direct, rapid diagnosis of the presence of anaerobic bacteria in specimens of pus taken from clinical sites (Gorbach et al., 1976; Phillips, Tearle and Willis, 1976). Both rely on the production of fatty acid metabolic products by anaerobes in vivo a characteristic not shared with facultative anaerobes or strictly aerobic organisms.

All positive findings were confirmed by subsequent isolation of obligate anaerobes from the specimens and only one false negative finding was reported from 98 specimens by Gorbach et al. (1976). Recent studies by Bricknell, Sugihara and Brook (1976) and others have shown that elevation of cerebrospinal fluid lactic acid occurs in cases of bacterial meningitis. Bricknell et al developed sensitive GLC techniques that were useful in diagnosis and particularly helpful in distinguishing between partially treated and aseptic meningitis.

Mitruka and his colleagues demonstrated that viruses could be detected and identified by means of gas chromatography (Mitruka and Alexander, 1968; Mitruka, Alexander and Carmichael, 1968; Mitruka, Norcross and Alexander, 1969; Mitruka, 1975). The viruses were differentiated on the basis of characteristic metabolic products produced in tissue culture media. Mitruka (1975) has reviewed the range of gas chromatographic applications in microbiology and medicine including the various approaches described in the literature for the identification of viruses, rickettsia, protozoa and helminths both in tissue cultures or clinical specimens and concluded that in selected cases the application of GC techniques had been shown to be a useful diagnostic aid but he noted the need for further research and the careful standardisation of procedures. The possible application of various gas chromatographic approaches to the detection and identification of viruses and

microorganisms in clinical specimens and the use of these techniques in clinical diagnostic service laboratories was also reviewed by Cherry and Moss (1969). The widespread availability of the VPI Anaerobe Laboratory Manual since 1972 together with other similar manuals (Sutter *et al.* 1972 and 1975) has encouraged the use of fatty acid end-product analyses as an aid to the identification of obligately anaerobic bacteria isolated from clinical specimens particularly in the United States (Moore, Cato and Holdeman, 1974). Moore *et al.* (1974) state that with the aid of chromatography to establish the genus fewer biochemical tests are required to speciate isolates. Finegold (1974) however had some reservations. He accepted that definitive identification of certain organisms might require analyses of metabolic products but pointed out that many clinical laboratories were not equipped with a gas chromatograph and were unlikely to acquire one in the near future. He considered that it might be desirable to work towards fulfilling this need or at least ensuring that such facilities were available in regional reference laboratories but stressed that it was necessary to develop a range of simplified tests, useful in clinical laboratories, that would enable accurate identification of the majority of isolates without recourse to gas chromatography. Similar views were expressed by Duerden *et al.* (1976).

The occurrence of Bacteroidaceae as commensals in man and animals

At or before birth and at all times thereafter Man and other animals are subject to bacterial contamination from their environment (Snyder, 1936; Haenel, 1961). The ability of a particular organism to colonise its host and become part of the normal flora is dependent upon a variety of factors. Members of the family Bacteroidaceae are one of the major groups of bacteria inhabiting the oral cavity and intestines of Man and other animals. The factors affecting colonisation of the human gut by these organisms have been reviewed by Drasar and Hill (1974) and they include physiological mechanisms, bacterial interactions and environmental influences such as the diet.

The human mouth is a complex structure containing a number of differing micro-environments each supporting a characteristic flora. Members of the family Bacteroidaceae are important commensals of the mouth occurring in the saliva, the gingival crevice and in plaque deposits on the teeth. The bacteriology of the mouth has been studied in detail and many of the factors influencing the establishment and maintainence of an oral microbiota have been elucidated. Comprehensive reviews of this subject have been given by Bowden and Hardie (1971), Hardie and Bowden (1974) and Holbrook (1976). The following Gram-negative anaerobic bacilli have

been isolated: B. oralis, B. melaninogenicus, B. ochraceus, F. polymorphum, B. corrodens, F. girans, F. naviforme and L. buccalis (Holdeman and Moore, 1973). The strains previously designated L. dentium have been shown to be Gram-positive aerobic organisms and they have been re-named Bacterionema matruchotti (Gilmour, Howell and Bibby, 1961).

Strains of the genus Bacteroides colonise the large bowel in very large numbers. Smaller numbers occur in the contents of the stomach and small intestine, being at the highest levels of about 10^4 or 10^5 organisms per gram wet weight immediately after a meal when the pH is highest. The numbers in the terminal ileum and at the ileo-caecal junction are higher than in other parts of the small intestine (Drasar, Shiner and McLeod, 1969). Although Bacteroides spp. are not known to be intimately associated with the intestinal mucosa they fulfil the criteria of autochthonous flora as defined by Savage (1972). It is possible that strains found in association with the mucosa of the large bowel (Nelson and Mata, 1970) are closely related (Tabaqchali, 1974).

Few studies have been made on the flora in the large intestine itself, as the majority of workers have studied the faecal flora (Drasar and Hill, 1974). Members of the family Bacteroidaceae have been isolated from the meconium of infants within a few days of birth (Williams Smith and Crabb, 1961; Deacon, 1973). Small numbers persist in

breast-fed infants, but they occur in higher numbers in the faeces of bottle-fed infants. Major increases occur in the counts of breast-fed infants after weaning (Snyder, 1940; Haenel, 1961). In the normal bottle-fed infant the number of bacteroides organisms present in the faeces equals or outnumbers the bifidobacteria (Haenel, 1970). Recent studies by Bullen and his colleagues have indicated that the nature of the breast-fed infant's faecal flora is influenced mainly by the presence of an acetic acid-acetate buffer system that disappears when supplementary feeding is introduced. The buffer system was not found in the faeces of bottle-fed infants (Bullen, Tearle and Willis, 1976). Breast-fed babies produced faeces with a low pH and high counts of saccharolytic bifidobacteria and streptococci. Bottle-fed babies and breast-fed infants receiving supplements produced faeces with a high pH and increased numbers of Gram-negative bacteria including Bacteroides sp. In vitro evidence suggested that the acetic acid-acetate buffer was produced by the metabolism of the saccharolytic bacteria in the large intestine and that the buffered acidic environment suppressed the growth of the Gram-negative putrefactive strains (Bullen and Tearle, 1976).

In the normal healthy adult, Bacteroides spp. are the predominant organisms in the large intestine and along with the bifidobacteria constitute more than 99% of the cultivable flora (Drasar, 1967; Drasar, Shiner and McLeod, 1969). Similar findings have been reported by various workers

(Moore *et al.*, 1969; Mata, Carrillo and Villatoro, 1969; Bhat *et al.*, 1972) and in subjects on very dissimilar diets (Peach *et al.*, 1974; Drasar, 1974; Ueno *et al.*, 1974).

Smith (1975) has stated that variations from one individual to another make it impossible to define the faecal flora except in general terms. Moore and Holdeman (1974) consider it likely that there may be as many as 500 species of bacteria in human faeces. The dominant recognised species include *B. thetaiotaomicron*, *B. vulgatus* and *B. distasonis* (Moore and Holdeman, 1974; Werner, 1974; Attebery, Sutter and Finegold, 1974). These closely related species, previously recognised as subspecies of *B. fragilis*, were present in counts of 10^9 to 10^{11} organisms per gram wet weight of faeces in virtually all the specimens examined by the different workers. *B. ovatus* and *B. fragilis* were common isolates in fewer specimens. Ueno *et al.* (1974) reported similar findings in both Japanese living on traditional diets and in Japanese and Americans living mainly on Western diets. In the Japanese on traditional diets, *Fusobacterium* sp. were also prevalent but these strains were either absent or present only in low numbers in the faeces of the subjects eating Western foods.

Many other *Bacteroides* spp. have been isolated from the faeces of healthy individuals and many strains do not belong to recognised species (Moore and Holdeman, 1974). Additional

recognised species have included B. melaninogenicus, B. capillosus, B. oralis, B. putredinis, B. coagulans and B. ruminicola (Holdeman and Moore, 1972). The recently named species B. splanchnicus was also isolated from faeces (Werner et al., 1975). In addition, strains commonly found in other animals, including B. hypermegas from poultry and B. trichoides from pigs have been occasionally isolated from human faeces (Peach et al., 1974).

Bacteroides species are generally regarded as commensals in the human vagina (Gorbach et al., 1973; Holdeman and Moore, 1972); however, these organisms were isolated from the vagina of only 15 of 280 (5.4%) pregnant women (Hurley et al., 1974). Smith (1975) has given a concise and interesting review of the anaerobic bacteria occurring as commensals in humans.

Members of the family Bacteroidaceae have been found as commensals in a wide variety of animals. Barnes and Impey (1970) found that these organisms formed almost 40% of the caecal flora of chickens. A common isolate was B. hypermegas (Barnes and Impey, 1971) and other strains isolated have included B. ovatus, B. thetaiotaomicron and F. necrogenes. Strains of the previously designated B. clostridiiformis were also isolated, but these organisms are now considered to be clostridia (Barnes and Goldberg, 1968) and have been reclassified as Clostridium clostridiiforme

(Kaneuchi et al., 1976; Cato and Salmon, 1976).

The intestinal and faecal flora of mice and rats has been studied in some detail. Williams Smith and Crabb (1961) reported that Bacteroides species were present in the faeces of 10 mice at average counts of 1×10^9 organisms per gram of faeces. Similar numbers were reported by Schaedler, Dubos and Costello (1965) who found that two different groups of Gram-negative anaerobic rods were established in these numbers about 16 days after birth. They were found to persist at these levels and remained almost exclusively in the large intestine. The organisms were not speciated, but descriptions given by Lee et al. (1970) were consistent with there being both Bacteroides and fusobacteria present. The fusobacteria in particular appear to be intimately associated with the mucous layers in the large intestine (Savage, 1971; Savage, McAllister and Davis, 1971). The influence of volatile fatty acids on the intestinal ecology of mice was investigated by Lee and Gemmell (1972). The ingestion of solid food by 11-day-old mice coincided with the appearance of volatile fatty acids and strictly anaerobic fusiform bacilli in the intestinal contents. The fusobacteria were shown to be responsible for the production of the volatile fatty acids, particularly n-butyric acid. Tannock (1977) has reported that the Bacteroides species he most frequently isolated from the caecum of conventional mice resembled

B. fragilis and B. thetaiotaomicron. The strains were not completely typical and differed in one or two biochemical characteristics. Other isolates included B. distasonis, but many were not typical of any recognised species. Holdeman and Moore (1973) reported that F. varium, F. russii, F. naviforme, B. distasonis and B. thetaiotaomicron are commensals of rodents. The same authors have named 5 species of Gram-negative anaerobic rods as commensals in pigs: B. distasonis, B. ruminicola, B. corrodens, B. capillosus and F. necrophorum. Holdeman and Moore (1974) have re-classified the previously recognised B. trichoides, isolated from pigs, as Clostridium ramosum although they state that spores are usually not seen.

The flora of the bovine rumen has been studied in detail by numerous workers. Much of the recent work was stimulated by improved techniques of anaerobic culture (Hungate, 1950). Detailed accounts of the flora found in the bovine rumen have been given by Bryant (1959) and Hungate (1966). Representatives of the family Bacteroidaceae have included B. succinogenes, B. amylophilus, B. ruminicola, B. nodosus, F. necrophorum and the following "strains of uncertain affiliation": Selenomonas ruminantium, S. lactilytica and Butyrivibrio spp.

Members of the family Bacteroidaceae have also been isolated from the body cavities of insects. B. termiditis

and B. niger are recognised species isolated from termites (Holdeman and Moore, 1974), and F. varium has been isolated from fish; in addition, B. serpens and F. aquatile have been isolated from contaminated or fresh water respectively.

The association of Gram-negative, non-sporing anaerobic bacilli with disease in man and animals

The Gram-negative anaerobic rods of the family Bacteroidaceae are frequently isolated in mixed culture from clinical specimens. The infections are often endogenous in origin; commonly the infected host has been compromised either by illness, immunosuppressive or antibiotic therapy, or by surgery, and is therefore more pre-disposed to infection by organisms present in the normal flora (Dowell, 1974). Merely finding these organisms under such conditions is not proof of their pathogenic role (Williams, 1974); however in humans, members of the family Bacteroidaceae have been recognised as significant pathogens in many clinical infections (Smith, 1975).

Finegold (1974) has reviewed the extensive literature on the incidence of these organisms in intra-abdominal, genito-urinary, skin and other soft-tissue infections. B. fragilis (B. fragilis ss. fragilis) is generally recognised as the most important pathogen in the group (Holdeman, Cato and Moore, 1974) and has been found in post-operative wound

infections by many workers (Gorbach, Thadepalli and Norsen, 1974). Finegold (1974) reported that strains of B. melaninogenicus and F. nucleatum have also been regularly isolated from such specimens. F. necrophorum has been found commonly in liver abscesses. B. fragilis is the species most commonly isolated from blood cultures (Fellner and Dowell, 1971) and has been the most frequent isolate from cases of neonatal or adult bacteriaemia (Chow and Guze, 1974). Nastro and Finegold (1973) reported that B. fragilis, F. nucleatum and F. necrophorum are infrequent causes of subacute bacterial endocarditis, and Fellner (1974) noted that the highest mortality occurred if B. fragilis was the causative organism. Strains of B. fragilis and B. melaninogenicus are the species most commonly isolated from pelvic infections in women (Thadepalli, Gorbach and Keith, 1973). In respiratory tract infections, particularly bronchiectasis and lung abscesses, F. nucleatum and B. melaninogenicus were frequently isolated, and strains of B. fragilis, B. oralis and F. necrophorum have been found regularly (Barlett, 1974).

Socransky (1970) and Hardie and Bowden (1974) have reviewed the extensive literature on the importance of bacteroides organisms in periodontal disease. B. melaninogenicus has been described as the primary pathogen in many oral infections of man (MacDonald, Socransky and Gibbons, 1963). Barlett (1974) considered that major disease syndromes of the upper respiratory tract involving anaerobic organisms

were now relatively infrequent. In earlier years Gram-negative anaerobic infections of the oropharynx often led to a severe tonsillitis or pharyngitis with associated thrombophlebitis and disseminated septic emboli in the lungs or joints. The more common, but less severe, chronic sinusitis, peritonsillar abscess and chronic otitis media are often linked with the isolation of B. melaninogenicus and Fusobacterium spp. normally present on adjacent mucosal surfaces.

There has been an increasing awareness of the pathogenic potential of bacteroides organisms in recent years, but despite improved anaerobic culture techniques there has been little progress in gathering experimental evidence of the pathogenic mechanisms involved (Rosenblatt et al., 1974). In part, this may be attributed to the fact that these organisms are frequently isolated from infections stemming from colonised mucosal surfaces and involving more than one organism, under these circumstances it is difficult to determine the precise role of each strain in the pathogenesis of the infection. A clear model was demonstrated by MacDonald, Gibbons and Socransky (1960) who found that B. melaninogenicus was an essential component in causing gingivitis. McDonald et al were able to produce subcutaneous infections in guinea pigs with gingivitis exudate only when these organisms were present. Infection could be induced when the exudate

contained B. melaninogenicus, another Bacteroides sp., a motile Gram-negative anaerobe and a facultative diphtheroid. Werner (1974) has stated that the pathogenic mechanisms associated with the virulence of the recognised pathogen B. fragilis are little understood; he listed as factors of possible significance, (a) endotoxic polysaccharides, (b) the capsule, (c) a neuraminidase, (d) a fibrinolysin, and (e) a haemolysin. Fibriolysin activity has not been found in B. thetaiotaomicron or B. vulgatus. The presence of a capsule has also been associated with the pathogenicity of B. melaninogenicus (Takazoe, Tanaka and Homma, 1971).

A possible role for Bacteroides spp. in the aetiology of carcinoma of the colon has been suggested by Drasar and Hill (1974). The commensal species in the gut can degrade bile acids and cholesterol and produce potentially carcinogenic compounds. The metabolism of the amino acid tryptophan by these species has also been linked with the production of carcinoma of the bladder (Drasar and Hill, 1974).

Members of the family Bacteroidaceae have been implicated in a variety of infections in animals. Smith (1975) has reviewed some of the more economically important of these. He cited the work of Deane and Jensen (1955) and Roberts and Egerton (1969) who established the essential role of B. nodosus and F. necrophorum in the aetiology and pathogenesis of foot rot in sheep. The combined role of B. melaninogenicus and F. necrophorum in the pathogenesis of

foot rot in cattle was established by Berg and Loan (1975). F. necrophorum has also been incriminated in "diphtheria" of calves, interdigital ovine dermatitis and hepatic abscesses in cattle (Smith, 1975).

Bacterial anaerobiosis

The existence of bacteria able to grow in the absence of air was first observed by Pastéur in 1861 (Smith, 1967) and the term anaerobes (Fr. anaérobies) has been used to describe these organisms. The capacity for anaerobic growth is now recognised as an attribute of many micro-organisms. Bacteria able to grow under ordinary atmospheric pressures of air or under conditions of reduced oxygen tension (pO_2) have been termed facultative anaerobes, whilst those that are unable to maintain vital metabolic activity in air have been termed obligate anaerobes.

Much difficulty and confusion has surrounded the precise definition of obligate anaerobes. Smith (1967) stated three commonly held definitions as follows:

"(i) microorganisms growing better in the absence of air than in its presence, (ii) bacteria that perish even on transient contact with atmospheric oxygen, or (iii) bacteria that are unable to initiate growth from small inocula unless the oxidation - reduction potential of the medium is low."

Smith regarded all these definitions as incomplete and only partial resolutions of the accumulated experimental

evidence. Other definitions have been based on modern concepts of energy-yielding metabolic processes. Decker et al. (1971) described obligate anaerobes as bacteria that do not possess either cytochrome oxidase or oxygenases to enable the utilisation of atmospheric oxygen as the terminal electron acceptor in the electron transport chain, and Morris (1975) regarded them as bacteria that (i) generate their energy and synthesise their substance without recourse to molecular oxygen, and (ii) demonstrate a singular degree of adverse oxygen sensitivity which renders them unable to grow under an atmosphere of air. None of these definitions is entirely satisfactory.

The anaerobic bacteria are a heterogeneous group with widely differing sensitivities to oxygen. Recently Loesche (1969) examined the ability of various obligate anaerobes to grow on solid agar and defined two types of anaerobe. Strict anaerobes were unable to grow on agar at pO_2 levels greater than 0.5% (e.g. Clostridium oedematiens type D), and moderate anaerobes, including members of the family Bacteroidaceae, were able to grow in oxygen concentrations of 2-8%. In addition Loesche (1969) found that moderate anaerobes were able to withstand exposure to atmospheric levels of oxygen for periods of 60-90 min. without appreciable loss of viability. He noted strain to strain variation. In discussing microaerophilic species, which have been regarded as a subgroup of the obligate anaerobes, he

concluded that this term best described those organisms that were able to grow better in 5-10% oxygen than under strictly anaerobic conditions. Berkeley and Campbell (1971) observed that some species such as Brucella abortus had been mistakenly termed microaerophilic when in reality they were facultative strains requiring increased concentrations of carbon dioxide.

The nature of bacterial anaerobiosis and the possible factors involved continue to be the subject of much investigation by numerous workers. The earlier hypotheses were concerned with (i) a direct toxic effect of atmospheric oxygen on the cells, (ii) the toxicity of intracellular or organic peroxides and the role of catalase in protecting microorganisms against these products, and (iii) the primary importance of a low redox potential (Eh) in culture media for the successful growth of demanding obligate anaerobes. More recently there has been a better understanding of the cellular and biochemical processes that may be affected by oxygen and its derivatives or a high redox potential. Six current hypotheses have been enunciated by Morris and O'Brien (1971) and by Morris (1975). They are: "(i) oxygen itself is the toxic agent and is lethal to the cell; (ii) normal growth and metabolism of the anaerobe is only possible within certain stringent limits of culture redox potential and the presence of free oxygen in the medium is incompatible with the attainment and maintainence of the low Eh values

required for growth. Reducing agents exhibit a protective action by virtue of their ability to poise the Eh value of a culture medium at a suitably low voltage or otherwise to assist the organisms to sustain the favoured redox state; (iii) the cell contains key components bearing free -SH groups (e.g. enzymes) whose oxidation by oxygen halts growth and metabolism; (iv) preferential reduction of oxygen unproductively consumes the cell's reducing power leaving insufficient for the cell's essential biosyntheses; (v) oxygen indirectly controls cellular activity by determining the intracellular concentration of a key metabolic regulator, presumably a participant in a redox couple liable to direct oxidation by oxygen or in equilibrium with another such couple; (vi) it is not oxygen per se but products of its utilisation that are toxic (e.g. superoxide anions and hydroxyl radicals and singlet oxygen) (Morris, 1975).

Detailed reviews of this complex field have been given by a number of workers (Smith, 1967 and 1975; Haugaard, 1968; Morris and O'Brien, 1971; Watt, 1972; Morris, 1975). The present author also reviewed this subject as part of his earlier studies (Deacon, 1973).

Methods of anaerobic culture

As a comparison of two up-to-date approaches to anaerobic culture is part of the experimental section of this thesis,

a detailed consideration of the technical aspects of these methods is included in the Discussion section; this part of the Introduction will be restricted to a brief survey of the historical aspects of the subject and general considerations of modern methodology. The evolution of anaerobic culture techniques was recently reviewed by Sonnenworth (1972). An earlier review by Hall (1929) stated the principles of anaerobic culture and described the early methods employed. Other reviews have been given by Willis (1964) and Watt (1972).

The aim of all the methods so far described has been to remove oxygen or reduce the oxygen tension in the culture environment and to maintain it at low levels throughout the period of incubation. Primary reduction has been accomplished by (i) biological systems, (ii) physical or (iii) chemical methods.

In the early days biological reduction was achieved by the addition of plant or animal tissues to liquid culture media or by the use of mixed cultures of facultative micro-organisms. Robertson's cooked meat broth, which is still widely used today, depends on the reducing conditions produced in the pink meat at the bottom of prepared tubes. The reducing systems in this medium were investigated by Lepper and Martin (1929) who found that the most important of these was the auto-oxidation of fatty acids present in the

lipins of muscle tissue. The reduction was catalysed by the haematin also present. A second system produced by the auto-oxidation of the amino acid glutathione was found to be of lesser importance.

Physical reduction was accomplished by boiling or presteaming of culture media; deep fluid or agar shake cultures were commonly used (Sonnenworth, 1972). Plate media were incubated under a vacuum or in an atmosphere of inert gas. Pasteur first introduced the use of an inert gas to remove oxygen from culture media with carbon dioxide (Hall, 1929) and later workers used hydrogen or illuminating gas.

Chemical reduction by the addition of reducing agents to the medium was first described in the 1890's (Hall, 1929). Early workers added substances such as glucose, sodium formate, sodium sulphonate, alkaline sulphides, ascorbic acid and sulphydryl compounds including thioglycollic acid, glutathione and cysteine. Cysteine hydrochloride was shown to be superior to cysteine by Hosoya and Kishino in 1925 (Sonnenworth, 1972). Laidlaw (1915) used spongy platinum to catalyse the combination of oxygen and added hydrogen in a closed air chamber. McIntosh and Fildes (1916) found that palladium was superior to platinum. Many workers used a variety of jars and bells as incubation chambers. Alkaline pyrogallol was the most frequently used reductant

in these devices, but some workers used iron compounds and phosphorus. Various single and double plate systems were developed again with pyrogallol as the reducing agent. The use of the agent has persisted to the present day, but it is not widely used now that more sophisticated techniques for the removal and exclusion of oxygen are available (Watt, 1972).

Many of the present-day anaerobic culture methods combine previously employed basic principles. In the roll-tube systems (Hungate, 1950 and 1966; Moore, 1966; Cato et al., 1970) primary reduction of media is accomplished by boiling and gassing with an oxygen-free gas mixture and a reducing agent is added to poise the redox potential at low levels before autoclaving. All subsequent handling procedures are then carried out under a stream of oxygen-free gas to maintain the pre-reduced and anaerobically sterilised media in an extremely reduced condition and to protect microbiological specimens and isolated strains from contact with oxygen. Pre-reduced and anaerobically sterilised media have been used in conjunction with anaerobic cabinet systems (Drasar, 1967; Aranki et al., 1969). Anaerobic cabinets or glove-boxes (Socransky, MacDonald and Sawyer, 1959; Rosebury and Reynolds, 1964; Drasar, 1967; Lee, Gordon and Dubos, 1968; Aranki et al., 1969; Leech, Bullen and Grant, 1971; Deacon, 1973; Deacon and Loutit, 1975) were also developed to allow the use of conventional plate culture techniques without exposing

specimens or freshly isolated bacteria to the potentially deleterious effects of oxygen at atmospheric pressures.

Anaerobic jar methods employing an evacuation and replacement approach for the removal of air have been improved by the development of standardised procedures (Collee et al., 1971; Collee, Watt, Fowler and Brown, 1972; Watt, 1972). Residual oxygen is removed by room temperature palladium catalysts in an atmosphere of 90% hydrogen and 10% carbon dioxide. Watt (1972 and 1973) showed that the addition of 10% CO_2 to the incubation atmosphere enhanced the growth of many anaerobic species on solid media. The use of three catalyst sachets was recommended by Watt, Hoare and Collee (1973) and a further improvement was the use of biological indicators of anaerobiosis (Watt, Collee and Brown, 1976). These workers and others have used reducing agents such as cysteine hydrochloride to poise the redox potential at low levels. Dithiothreitol, an artificially developed thiol compound (Cleland, 1964), has been used in combination with cysteine hydrochloride by Moore (1968) and by Watt (1972) and others. It seems that dithiothreitol is not easily auto-oxidised by contact with air and can be used to protect the more labile cysteine hydrochloride.

A simple alternative to the evacuation and replacement methods used with anaerobic jars has been developed for use in clinical laboratories. The Gaspak system (BBL) employs a foil sachet containing sodium borohydride and

citrate-bicarbonate tablets that release hydrogen and carbon dioxide on contact with water (Brewer and Allgeier, 1966). Water (10 ml) is added to an open sachet immediately before placement in an anaerobic jar. The gases are released in the sealed jar in proportions of approximately 90% hydrogen and 10% carbon dioxide. The hydrogen reacts with the palladium catalysts to remove oxygen and produce the reduced conditions. The system has been evaluated in comparison with a standardised evacuation-replacement technique by Collee et al. (1972) and by Dowell (1972). Reservations were expressed by Ferguson, Philips and Tearle (1975) and a new development (the Gaskit, Oxoid) has just been launched on the market.

Some workers, concerned that media may become inhibitory to growth of demanding anaerobes if stored in the presence of oxygen, have recommended the use of fresh or anaerobically stored media (Aranki et al., 1969; Dowell, 1970; Finegold, 1970; Holdeman and Moore, 1972). Greater attention is now being paid to the collection and preservation of clinical specimens as this is recognised as an important part of the success or failure of any anaerobic procedure (Dowell, 1974).

MATERIALS AND METHODS

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The Materials and Methods section of this thesis is divided into five sections: (A) General procedures, (B) Handling procedures and redox potentials in liquid culture media, (C) Growth studies on *B. melaninogenicus* ss. *asaccharolyticus*, (D) Gas chromatography in the identification of the Bacteroidaceae and (E) Identification and characterisation of Bacteroidaceae.

SECTION A

GENERAL PROCEDURES

Preparation of culture media

Basal media, not containing any heat-labile substances, were sterilised by autoclaving at 103.5 kilopascals for 15 min. All heat-labile substances were added prior to inoculation of liquid media or prior to pouring of plate media. Sterile, basal liquid media were either freshly prepared or taken from storage at 4°C within 2 weeks of preparation, steamed for 30 min. before use to remove dissolved oxygen and cooled rapidly to 37°C before addition of heat-labile substances and inoculation. Solid media were freshly prepared or stored at 4°C and used within 2 days of preparation.

Blood agar (BA) plates contained Columbia agar base (Oxoid) and 5% outdated human blood. The blood was obtained from the Regional Blood Transfusion Service, Edinburgh. Each 500 ml of blood contained 2 g disodium citrate and 1.7 g of dextrose in 70 ml of water; lysed blood was prepared by treatment with saponin prior to incorporation of the blood preparation into the sterile basal medium. The formulae and details of preparation of all media used in the study are included in Appendix I.

Pre-reduced anaerobically sterilised liquid media (PRAS). Media for use in the anaerobic cabinet were prepared by the techniques first described by Hungate (1950) and subsequently modified by him (Hungate, 1966). Unless otherwise stated

PRAS media were dispensed in 10 or 20-ml quantities in 25-ml screw-capped McCartney containers fitted with new rubber liners. The media contained 0.00001% of resazurin, a redox indicator that remains colourless below an Eh of -50 mV at pH 7.0 (Twigg, 1945).

Heat-labile culture supplements

Filter-sterilised aqueous stock solutions of the following growth factors were added aseptically to all steamed and cooled liquid media: haemin (haematin hydrochloride, BDH) 500 µg/ml, and menadione (vitamin K_3, Sigma Chemicals) 100 µg/ml, to give final concentrations of 5.0 ug/ml and 1.0 µg/ml respectively (see Barnes and Impey, 1971); 3.75% cysteine hydrochloride (BDH) and 4.0% Na_2CO_3 to give final concentrations of 0.075% and 0.04% v/v respectively. Carbohydrates required in some media were prepared as filter-sterilised 20% stock solutions and added to basal media to give a final concentration of 1% of the test substrate. A range of pure amino acids, vitamins, salts and Tween 80, used in some of the media trials, are listed in a later section. Details of the preparation and sterilization of all supplements used in these studies is given in Appendix I.

Culture inocula

Unless otherwise stated in the text, one drop (c. 0.02 ml) of a 48-h CMB culture was used routinely to inoculate tubes and either one drop or one loopful was used to inoculate plates.

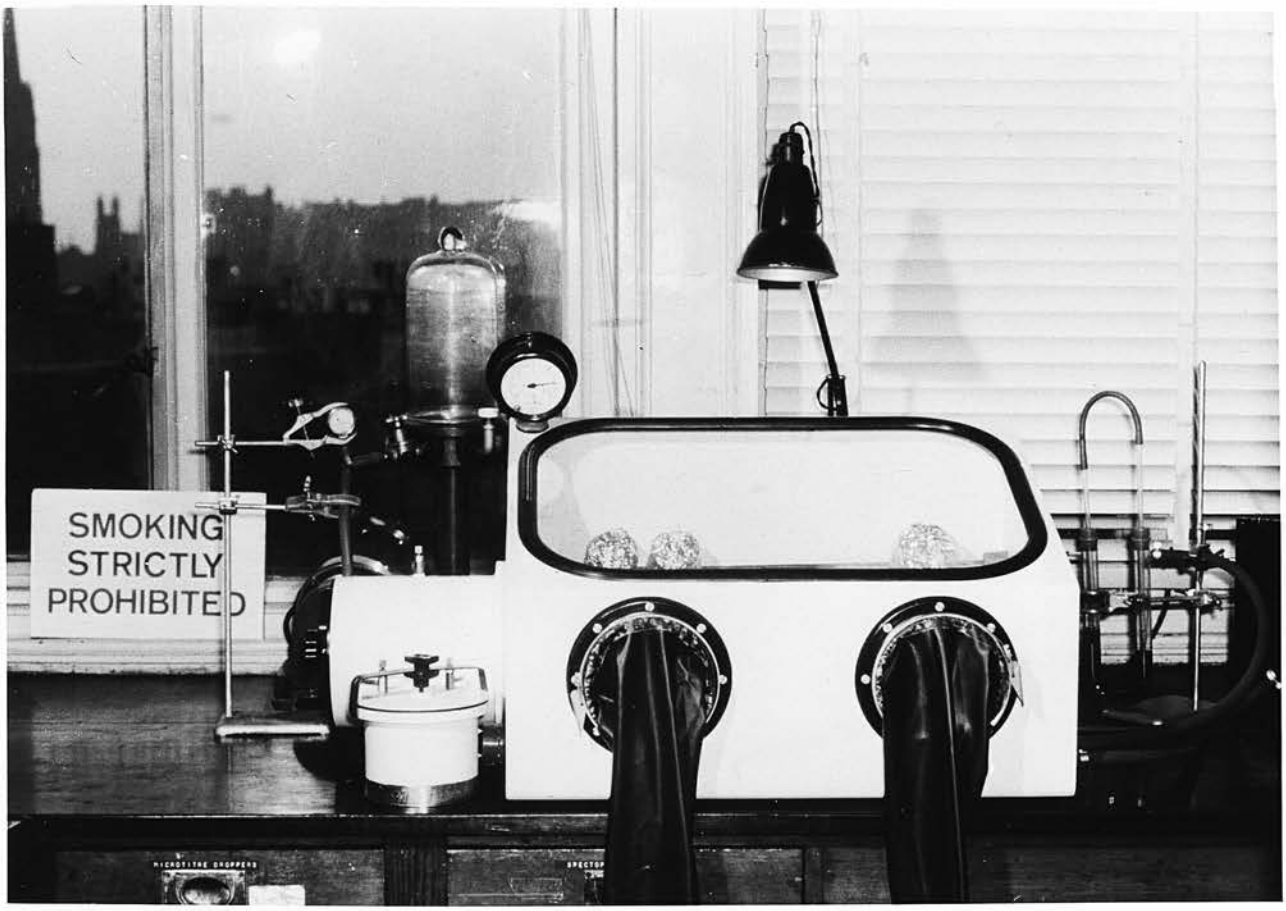
Standard anaerobic culture procedure

Baird and Tatlock Ltd (BTL) anaerobic jars were used throughout the study; they were fitted with 3 room-temperature-active catalyst sachets. Chemical indicators of anaerobiosis were not used and the side arms were sealed off (Collee, Rutter and Watt, 1971). The standard anaerobic procedures of Collee et al. (1972) were used throughout the study with a single cylinder of 90% H_2 and 10% CO_2 (BOC Ltd) replacing the two separate cylinders originally described. All incubations were at 37°C.

Anaerobic cabinet procedures

The design and construction of the anaerobic cabinet (plate 2) used in these studies has been described by Watt, Collee and Brown (1973). The setting up procedure prior to routine operation has been modified. For the present study, most of the air present in the cabinet was removed by placing a lighted candle in the cabinet (Drasar, 1967) and a slight positive pressure was maintained with a trickle flow of CO_2 gas until the candle was extinguished. The cabinet was then flushed for 3 days with a gas mixture of 3% H_2 in 97% N_2 (1.0 litre/min.) and pure CO_2 at a flow rate of 100 ml/min. giving a final concentration of 10% (v/v). The gas mixture was passed through a De-oxo catalytic purifier (Englehard Industries Ltd) to remove all traces of oxygen before entry into the cabinet. Five catalyst sachets were then passed into the cabinet through the airlock and flushing continued for a further 3-4 days. Routine operation of the cabinet

Plate II. The anaerobic cabinet



The cabinet measures 60.5 cm x 45 cm x 45 cm at maximum height and has a volume of 110 l; a cylindrical transfer port of volume 5.7 l is attached on the left side and gas supply and exhaust tubes are on the right. Reproduced from Watt et al. (1974) with permission.

followed the procedures of Watt et al. (1973). The use of biological indicators of anaerobiosis was continued. In addition, a potentiometric device for measurement of redox potentials in culture media (described below) was adapted to monitor the exhaust gases continuously from the cabinet to ensure that an extremely reduced oxygen-free environment was maintained throughout all manipulations (for detailed description, see p. 64).

Maintainence of cultures

All strains examined in this study, whether fresh isolates, cultures referred by other workers or type culture and reference collection strains were preserved as freeze-dried cultures in the laboratory. Stock cultures were grown in Robertson's cooked-meat broth (CMB; Cruickshank, 1968) for 48 h in an anaerobic jar and held anaerobically for up to 2 weeks on the bench. Cell morphology and culture purity were checked regularly by wet films with phase contrast microscopy, by Gram-stained smears, and by aerobic and anaerobic subculture on Columbia agar (Oxoid) plates containing 5% human blood.

pH measurement

Unless otherwise stated, a Pye Unicam 292 pH meter with a combined test/reference electrode was used.

Microscopy

A Carl Zeiss RA38 research microscope equipped with phase-contrast objectives and substage condenser was used

for all microscopy in this study. Total counts were performed with a 0.1-mm depth Hawksley counting chamber with Thoma ruling and followed the procedures described by Collee, Rutter and Watt (1971).

SECTION B

HANDLING PROCEDURES AND REDOX POTENTIALS IN LIQUID CULTURE MEDIA

In proceeding to investigate the possible causes of growth failures with some strains in the culture media used in the characterisation of clinical isolates it was necessary first to re-examine some aspects of the handling procedures in use in the laboratory before embarking on an extensive study of the nutrient requirements, in complex media, of our isolates.

Eh measurements in Robertson's CMB medium

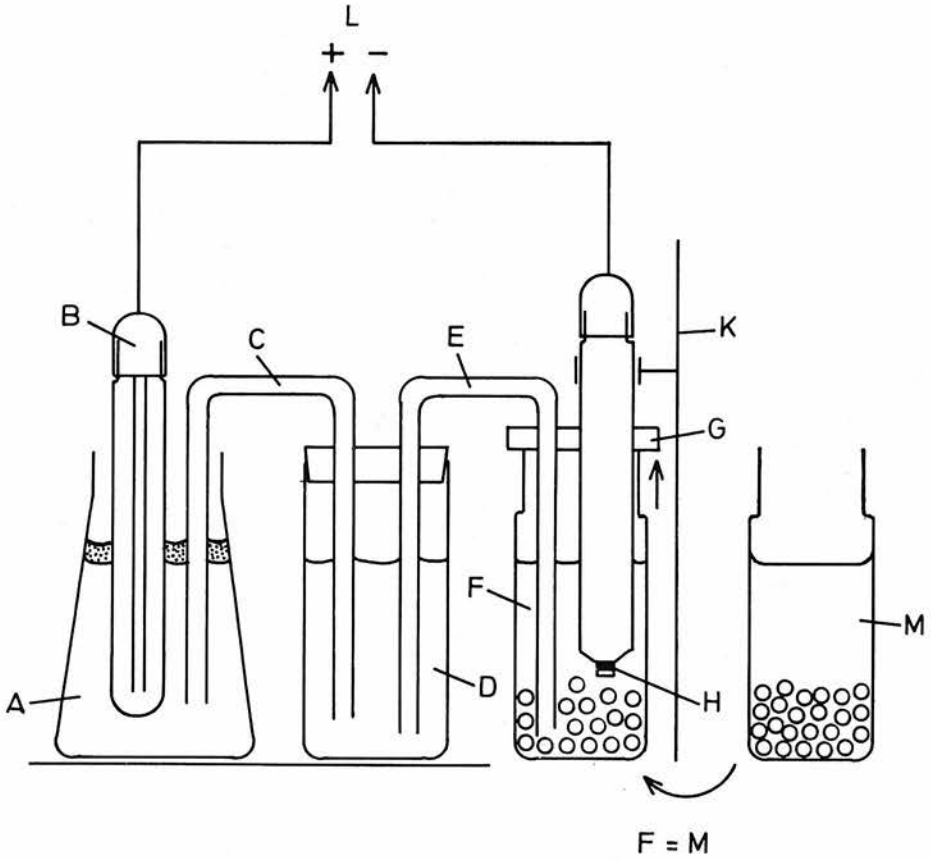
A flawless, polished platinum ring electrode (Pye-Ingold No. 805) was employed to measure the redox potentials (Eh) in 20-ml quantities of Robertson's cooked-meat broth (CMB) both before and after steaming for 30 min. in order to show that pre-steaming of broth before inoculation is an effective means of removing dissolved oxygen and hence provides adequately reduced conditions for rapid and reliable initiation of growth by obligate anaerobes.

Preparation of the test medium. The CMB medium used in this laboratory is normally prepared and dispensed in 10-ml quantities in test tubes fitted with loose-fitting metal caps. For this experiment, a total of six 20-ml quantities of CMB medium were prepared by pooling. Each 20-ml quantity was prepared by transferring with aseptic precautions the contents of two 10-ml tubes into one 27-ml screw-capped universal container. The universal containers were found

to be sufficiently wide (2 cm) to accept the platinum electrode and its accessory salt-agar bridge. After pooling, the CMB broths in universal containers were left on the bench with caps loose for 24 h to equilibrate before the first readings were taken. The broths were then steamed for 30 min. at 100°C with caps loose. On removal from the steamer the caps were tightened and the broths cooled rapidly to room temperature before the second readings were taken.

Setting up the apparatus. The apparatus was set up as in fig. 1. The procedures were adapted from Hewitt (1950), incorporated ideas of Vennesland and Hanke (1940), and followed the recommendations of Jacob (1970). The reference electrode used was a calomel half-cell ($Hg/HgCl_2$/saturated KCl; Pye-Ingold No. 303). An Erhlenmeyer flask (A) contained the reference electrode (B) immersed in saturated KCl covered with a layer of mineral oil to prevent excessive evaporation and crystallisation. The bridge (C) was prepared separately by filling a 4-mm i.d. glass U-tube with 2% molten agar in saturated KCl and allowing it to solidify at room temperature. The reservoir (D) contained sterile 1.0% NaCl in 2.0% agar and a 4-mm i.d. flexible clear plastic tube (E) filled with the same electrolyte. The tube connected the reservoir electrically to the 27-ml universal (F) containing the broth. During autoclaving, the reservoir was fitted with a rubber bung carrying an air bleed tube in place of the tube E. After sterilisation, one end of the tube was inserted through

Figure 1. A diagrammatic representation of the apparatus used to measure the redox potentials in samples of Robertson's cooked meat broth.



Key

A = a 250-ml Erhlenmeyer flask containing saturated KCl and an overlay of oil to prevent re-crystallisation.

B = the calomel reference electrode (Pye Ingold No 303).

C = 4-mm i.d. glass tubing bridge containing saturated KCl and 2% agar.

D = reservoir containing NaCl 1% in agar 2%.

E = flexible plastic bridge containing NaCl + agar.

F = 20 ml of Robertson's CMB in a 20-oz universal container (see also M).

G = rubber collar.

H = platinum ring electrode (Pye Ingold No 805).

K = stand and clamp.

L = Pye Dynacap pH/mV electrometer connections.

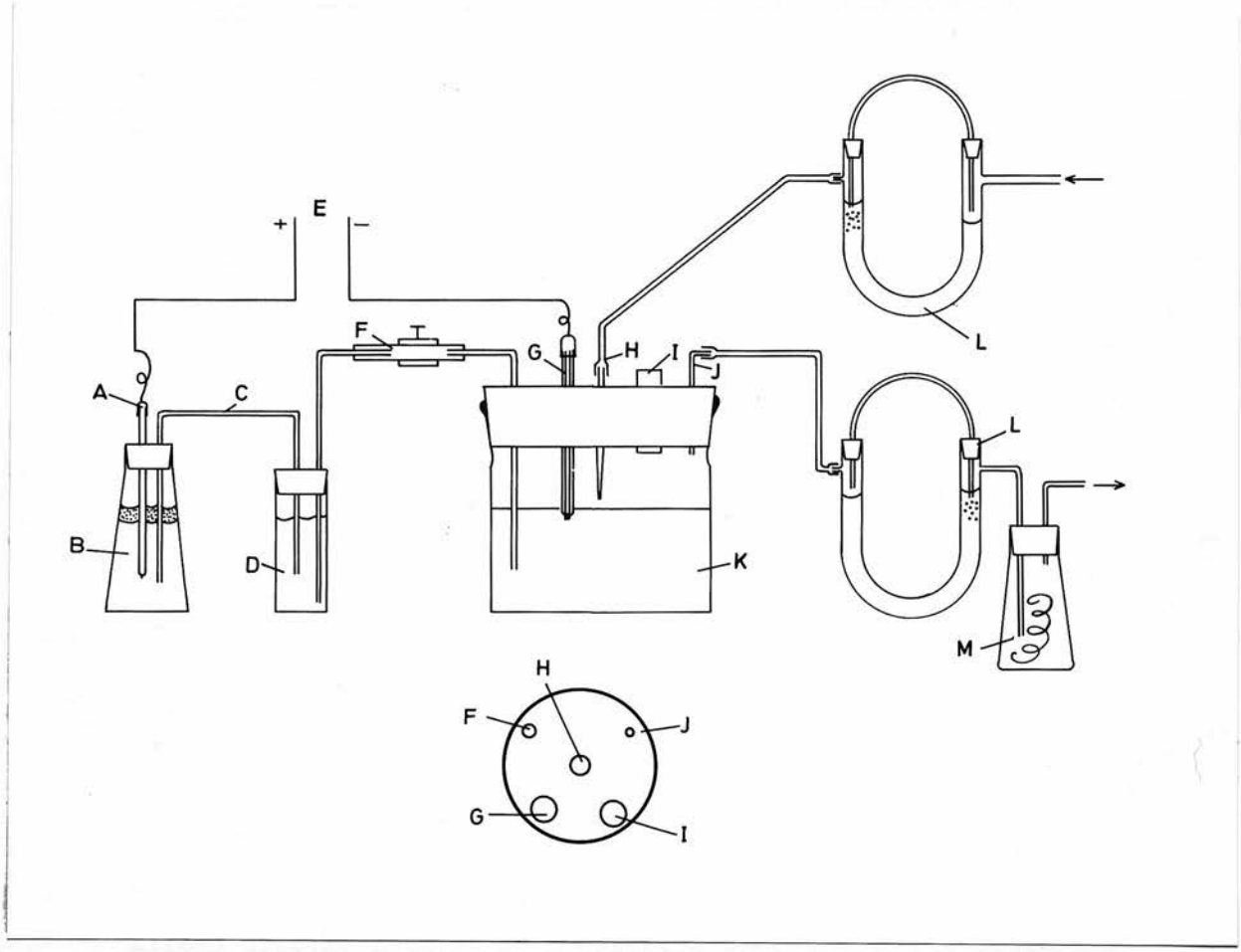
the bung and filled with molten agar by suction with a rubber bulb which remained in place until the agar had solidified. The tube was then fitted with the rubber collar (G) carrying the platinum electrode (H). The electrode was polished with a soft clean cloth and metal polish (Jacob, 1970). The electrode and the exterior of the bridge were sterilised by careful wiping with a cotton swab soaked in diethyl ether followed by rinsing in sterile distilled water, and then it was immersed in sterile nutrient broth until required for use.

Operation. The 2 electrode leads were connected to the anode and cathode sockets of the Pye Dynacap pH/mV electrometer. Initially the Pt electrode was connected to the cathode and the instrument zeroed. On transfer of the probes into the aerated CMB test broth, from the sterile nutrient broth, an off-scale deflection indicated a strongly positive Eh reading; the electrode leads were then switched around to obtain an on-scale deflection. Test readings were taken 180 seconds after switching. During a series of readings when the leads were not changed about, the readings were taken 180s after transfer of the probes into a test broth (Jacob, 1970). Readings were taken with the Pt electrode immersed to a depth of 2.5 cm in 20-ml quantities of broth having a full depth of c. 5.0 cm. During the experiments some readings were also taken with the electrode immersed in the fresh meat particles at a depth of c. 4.5 cm. The meat particles occupied the lower third of the universals.

Apparatus for continuous monitoring of anaerobic cabinet exhaust gases

Setting up and operation. The complete apparatus is shown in fig. 2. A 400-ml beaker containing 200 ml of Trypticase Soy broth (BBL) was fitted with a rubber bung carrying gas inlet and outlet tubes, 6 mm i.d., plugged with cotton wool. The inlet tube was tapered internally and sited 4 mm above the medium; the outlet tube was bent at right angles above the bung. A bridge fitted with a screw-clamp was inserted into the bung and connected to a 27-ml universal container that contained 20 ml of 1% NaCl in 2% agar (after Vennesland and Hanke, 1940). Holes in the bung for pH and Eh (Pt) electrodes were closed off for sterilisation with close-fitting glass stoppers. For autoclaving, the bung and its tubes were covered with aluminium foil. The rim of the bung was taped with autoclave tape to prevent adhesion to the beaker. During sterilisation the screw clamp on the bridge remained closed, air was forced out from both sides and replaced by medium and molten agar. After sterilisation, the apparatus was cooled on the bench and the bung firmly seated and then sealed to the beaker with candle wax to ensure a gas-tight fit. The Pt electrode was polished and sterilised separately, as described earlier, before being placed in position with the Pt ring 4 mm below the surface of the medium. Before the agar in the bridge and universal container had solidified, the other half of the measuring

Figure 2. A diagrammatic representation of the apparatus designed to monitor continuously the anaerobic cabinet exhaust gases.



Key

A = the calomel electrode.

B = saturated KCl in a 250-ml Erhlenmeyer flask.

C = the glass bridge.

D = the reservoir.

E = the electrometer connections.

F = bridge and screw clamp.

G = the platinum electrode.

H = gas inlet.

I = site for pH electrode: solid glass bung in place.

J = gas outlet.

K = 200 ml of Trypticase Soy broth (BBL) in a 400-ml beaker.

L = non-return oil seal. M = gas purifier.

The setting up and operation of the apparatus is described in the text.

circuit was set up as shown in fig. 2. When ready for use, the screw-clamp was released to complete the electrical connection of the monitoring vessel to the reference half-cell. During normal operation it was found that a pH electrode was not required and the glass stopper remained in place. Provision for the electrode was originally made to allow monitoring of the pH changes in the medium during gassing with the cabinet gas mixture containing CO_2. Rubber tubing connected the monitoring apparatus to the cabinet gas exhaust system via a U tube-oil trap system; a similar U tube-oil trap was connected to the gas outlet of the monitoring vessel.

Description of oil seal operation. This apparatus was designed by Mr R. Brown to control the pressure within the cabinet and the monitoring vessel. At zero pressure both ends of the glass tubing (X and Y) are under the surface of the oil. The height of the tube X, distal to the gas inlet, allows precise setting of a positive pressure in the anaerobic cabinet. For normal operation a pressure of +1 inch of water is maintained. When gas flow from the supply cylinders into the cabinet ceases, the oil seals the outlet X and maintains the cabinet pressure. A negative pressure of 1" must be applied before air will be sucked back through the seal contaminating the cabinet atmosphere. The second seal fitted beyond the monitoring vessel is similarly adjusted.

Experiments to show the effectiveness of the cabinet monitoring system

1. Aim. To show the change in the redox equilibrium when a reduced medium is briefly aerated.

Method. The monitoring system was set up as shown in fig. 2 except that a simple Y joint was inserted into the inlet tubing near the monitoring vessel. One arm of the Y carried the incoming gas mixture and the other was fitted with a short length of rubber tubing clamped off with a screw clamp. In practice, aeration was achieved by slowly releasing the screw clamp until air was drawn in and mixed with the O_2-free gas. The gas mixture for this experiment was led directly from the De-oxo catalytic purifier and bypassed the anaerobic cabinet. The inlet tube contained a sterile cotton wool filter; preliminary trials had shown that under these conditions contamination of the medium from the gas supply or tubing did not occur.

Gassing was commenced while the monitoring vessel and medium were still hot after autoclaving. The flow rate was approximately 1.0 litre/min. When the medium had cooled to room temperature and the E_{Pt} was -580 mV ($\pm$ 5) the medium was aerated for 30 seconds and O_2-free gassing was then continued.

2. Aim. To follow the changes with time when an aerated medium is subjected to a flow of O_2-free gas, re-aerated briefly and then again subjected to O_2-free gas.

Method. The apparatus was set up as described above. In this experiment the monitoring vessel and medium was allowed to cool on the bench without O_2-free gassing. When cool, the first electrometer readings were taken with the Pt electrode connected to the anode and the reference to the cathode. Gassing at 1.0 litre/min. was then commenced and within 5 min. the E_{Pt} had fallen to almost zero at which point the electrodes were interchanged at the cathode and anode inputs. All subsequent readings were taken with the electrodes undisturbed. Oxygen-free gassing was maintained for 180 min.; the medium was then aerated for 4 min. before O_2-free gassing was resumed. The experiment was continued for 290 min.

Comparison of anaerobic cabinet and standard bench procedures

A range of obligately anaerobic reference strains, representing 5 different genera, were subcultured into a liquid medium on the bench and in the anaerobic cabinet to compare the population densities obtained after 48 h and 72 h anaerobic incubation.

Organisms. The following 6 strains, held in the laboratory collection, were recovered from freeze-dried ampoules in Robertson's CMB for this study: Bacteroides fragilis NCTC 9343; Clostridium tetani NCTC 5405; C. oedamatiens, type D, NCTC 8350; an Anaerobic coccus NCTC 11-9801; Fusobacterium polymorphum NCTC 10562; and Leptotrichia buccalis NCTC 10249.

Medium and inoculum. Conventionally prepared PYG medium (containing the balanced salts solution of Holdeman and Moore, 1972) was used in conjunction with standard bench procedures. The same medium, pre-reduced and anaerobically sterilised, was used in the anaerobic cabinet. Methods of preparation were as detailed in section A of the Materials and Methods; the formula of the medium is given in Appendix I. Duplicate tubes of the two versions of the medium were inoculated with 0.02 ml and 0.04 ml of a 48-h CMB culture of each test strain. Cultures were incubated for 2 days in anaerobic jars with 10% CO_2. The degree of growth was recorded and the cultures re-incubated for a further day when final results were recorded.

Recovery and subculture of exacting strains

A further comparison of the bench procedures of Collee et al. (1972) and the anaerobic cabinet was conducted with three strains kindly supplied by Drs Ella Barnes and Clive Impey of Norwich. These strains were considered by Drs Barnes and Impey to be especially demanding in their requirement for strictly anaerobic conditions during handling and for the provision of extremely reduced conditions for initiation of growth.

Organisms. The strains studied included the type strain of Bacteroides ruminicola ss. brevis GA 33 originally isolated from rumen contents by Bryant et al. (1958) and two strains of Gemmiger formicilis (Gossling and Moore, 1975) including the

type strain ATCC 27749 (originally numbered VPI X_2- 56-1). This strain was isolated by Gossling and Moore from human faeces. The second strain of G. formicilis NE3/247 was isolated from a chicken caecum.

Media. Three media were used in the study including i) Robertson's CMB, ii) BM broth (modified from Williams et al., 1975) enriched with fresh meat particles (BM-CM), and iii) VL medium (Barnes and Impey, 1971). Detailed formulae are given in Appendix I. In addition VL agar and blood agar plates were used for purity checks and were inoculated in the anaerobic cabinet.

For use with the standard bench technique the CMB and the BM-CM media were prepared in the usual manner, and dispensed in 10-ml quantities in 6 x 5/8 test tubes but the VL medium was dispensed in 19-ml quantities into 1-oz McCartney bottles. The normal heat-labile culture supplements, detailed in section A, were added to the CMB and BM-CM media after pre-steaming and before inoculation. The haemin, liver and faecal extracts detailed by Barnes and Impey was added to the pre-steamed VL medium immediately before inoculation. This mixture consists of 1 part haemin at a concentration of 40 µg/ml, 2 parts of Difco liver extract, and 2 parts of faecal extract (detailed by Barnes and Impey, 1971). For use, 2.5 ml are added to 19 ml of basal medium.

All media for use in the anaerobic cabinet were dispensed in 19-ml quantities in 1-oz screw-capped McCartney bottles fitted with new rubber liners. The VL medium was pre-reduced and anaerobically sterilised by the methods detailed in section A of the Materials and Methods. The CMB and the BM-CM media were prepared conventionally in the media kitchen. Prior to passing into the anaerobic cabinet they were steamed and promptly cooled, and the heat-labile culture supplements were added. After transfer into the anaerobic cabinet the caps were loosened and the tubes left undisturbed for at least 48 h to allow complete reduction in the cabinet atmosphere.

Method. The anaerobic cabinet was set up as described previously with the exhaust gas monitor in operation. The freeze-dried ampoules containing the test strains were opened inside the cabinet and the contents of each reconstituted in 1 ml of PRAS VL medium. One drop (0.02 ml) of each was seeded into each of the 3 media and on to VL agar and blood agar plates. The remainder of each sample was placed in a sterile 7-ml screw-capped bijoux bottle and passed out of the cabinet. The broth media and the VL agar plates were placed in an anaerobic jar within the cabinet, sealed and passed out of the cabinet for incubation. The BA plates were passed out of the cabinet and incubated aerobically. The samples in the bijoux bottles were used to inoculate the conventionally

prepared media using the standard anaerobic procedures detailed earlier. Again, the BA plate was incubated aerobically and all other media were incubated anaerobically in jars at 37°C.

All broths were examined after incubation for 2 and 4 days. Wet films and Gram-stained smears were examined as purity checks. Each culture showing pure growth was subcultured into fresh medium after incubation for 4 days. At each passage, BA plates were inoculated and incubated aerobically as purity controls.

SECTION C

GROWTH STUDIES ON BACTEROIDES MELANINOGENICUS ss. ASACCHAROLYTICUS

During the development of characterisation procedures for general application to the Bacteroidaceae, preliminary studies revealed problems in the culture of some exacting strains. B. melaninogenicus ss. asaccharolyticus provides a good example and this subspecies was chosen as a model for growth studies.

Organisms

The source of each strain examined in this section is given in table 2. Bacteroides strains belonging to species or subspecies other than B. melaninogenicus ss. asaccharolyticus were included in some experiments for comparison.

Assessment of growth in liquid media

Growth in liquid cultures was routinely assessed either by direct visual observation or spectrophotometric readings. Visual assessments on well mixed cultures were graded on a scale of $\pm$ (just visible turbidity) to 5+; the 5+ grading was reserved for cultures showing very dense growth. All observations were made with the 6 x 5/8 in. test tubes used routinely in the laboratory. The assessments were made carefully with transmitted natural light and all assessments were initially checked by a second observer when establishing the grading system. Wet film preparations from tubes without visible turbidity or from any with a $\pm$ grading were checked by phase contrast microscopy to detect and accurately assess low levels of growth.

Table 2: The source and identity of all strains tested in the growth studies on B. melaninogenicus ss. asaccharolyticus

Strain	Subspecies	Source
NCTC 9337	B. melaninogenicus ss. asaccharolyticus	National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale, London.
Bangour 2296	" " " "	Dr R. Wiseman, Bangour General Hospital
Bangour 3502	" " " "	
Bangour 3586	" " " "	
WPH 34	" " " "	Edinburgh Dental Hospital
36	" " " "	
37	" " " "	
41	" " " "	All High Vaginal Swab (HVS) isolates obtained from the Royal Infirmary, Edinburgh.
44	" " " "	
45	" " " "	
94	" " " "	
Other strains tested for comparison (controls)		
NCTC 9336	B. melaninogenicus ss. intermedius	NCTC
NCTC 9338	" " " "	NCTC
ATCC 15930	B. oralis (B. melaninogenicus ss. melaninogenicus)*	American Type Culture Collection (ATCC)
VPI 4196	B. melaninogenicus ss. melaninogenicus	Dr E. Cato, Virginia Polytechnic Institute and State University (VPI), Blacksburg, Va. 24060, U.S.A.
NCTC 9343	B. fragilis (Cato & Johnson, 1976)	NCTC
NCTC 9344	" " (" ")	NCTC
NCTC 10582	B. thetaotaiomicron (" ")	NCTC
NCTC 10583	B. vulgatus	NCTC

* See Holbrook and Duerden (1974).

ATCC 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A.

When spectrophotometric assessment of culture turbidity was required the absorbance of well mixed cultures was read in 1.0-cm cuvettes, against distilled water, at 600 nm in a Pye Unicam SP 600 spectrophotometer. The absorbance of an uninoculated sample of each test medium was read with each batch of cultures and the test readings corrected accordingly. Unless otherwise stated, any sample with an absorbance reading over 1,000 was diluted 1 in 3, or more if necessary, with distilled water and read against an uninoculated control sample similarly diluted. Results were then multiplied by the dilution factor to obtain the correct figure. Duplicate or triplicate tubes were inoculated for each test sample in all growth experiments where turbidity readings were taken and the results were collated.

Correlation of culture turbidity and total cell count

A single experiment was performed to establish the grading system for visual assessment of culture turbidity and to establish the correlation of culture turbidity, as measured spectrophotometrically, with total cell counts on 3 reference strains of *Bacteroides* spp. and 1 strain of *Fusobacterium polymorphum*.

Organisms. The test strains were *B. fragilis* NCTC 9344, *B. melaninogenicus* ss. *intermedius* NCTC 9336, *B. melaninogenicus* ss. *asaccharolyticus* NCTC 9337 and *Fusobacterium polymorphum* NCTC 10562.

Method. The test strains were recovered from lyophilised ampoules and incubated for 48 h in Robertson's CMB medium. After purity checks, total cell counts were performed on 100-fold dilutions of the CMB cultures and 0.02 ml of the undiluted cultures were used to inoculate 10-ml volumes of PPYSG medium. After incubation for 48 h, the PPYSG cultures were mixed well and doubling dilutions made in PPY medium containing 0.02% formalin. The absorbance of all tubes was read at 600 nm against uninoculated PPY medium. Dilutions with an absorbance $\geq$ 1.000 were further diluted to give an absorbance below 0.500 and the results corrected accordingly. Total cell counts were performed on the 1 in 128 dilutions of each test strains. Part of the PPYSG culture of B. fragilis NCTC 9344 was also diluted to prepare a series of tubes representative of the 6 empirically selected categories of visual turbidity ($\pm$ and 1+ to 5+). The absorbance of each tube was read and recorded to provide a permanent reference.

Additions to PY medium

In the preliminary studies some strains failed to grow when subcultured into the PY or PYG media described by Holdeman and Moore (1972). A range of possible growth factors were added to PY medium in an attempt to improve this medium.

Supplements. These are detailed in table 3. Each was prepared as a sterile stock solution and added to the given concentration immediately before inoculation of the presteamed and cooled basal medium. Haemin, menadione and cysteine HCl were added to all media but sodium carbonate was replaced in PY medium by the sodium bicarbonate incorporated in the balanced salts solution of Holdeman and Moore (1972). Duplicate tubes of the complete PY medium, without test supplements, were inoculated with each set of cultures. Visual assessments of growth, confirmed where necessary by wet film examination, were made after incubation for 2 days; the cultures were then reincubated for a further 3 days when final results were recorded.

Inoculum and test strains. 0.04 ml of a 48-h CMB broth culture of four fresh clinical isolates of B. melaninogenicus ss. asaccharolyticus were used to inoculate the test media. The Bangour strains 2296 and 3502 and WPH strain 44 were tested with all the supplements; Bangour strain 3586 was tested with some.

Growth of B. melaninogenicus ss. asaccharolyticus in enriched culture media

The following series of media trials were undertaken to study the growth of B. melaninogenicus ss. asaccharolyticus strains in a range of rich, complex, liquid culture media and to select media suitable for use in the characterisation of clinical and reference strains of Bacteroides spp. Media

Table 3: Growth studies on B. melaninogenicus ss. asaccharolyticus; supplements tested for possible growth enhancement in PY medium*

Supplement (and stock concentration)	Final concentration
DL methionine (BDH) 74.0 µg/ml	7.4 µg/ml †
6.0 mM $(NH_4)_2SO_4$ + 0.04% (w/v) $FeSO_4.7H_2O$ (BDH)	5.0% (v/v) and 0.0004% †
glutathione (BDH) 10.0 mg/ml	100 mg/litre
pyruvic acid $CH_3.CO.COOH$ (BDH)	1.0 ml/litre
L tryptophan (BDH) 1.0 mg/ml	10 mg/litre
disodium succinate (Sigma Chemicals) 50.0 mg/ml	50 mg/100 ml
0.880 ammonia (BDH)	0.02% (v/v)
Horse serum (Wellcome Laboratories)	2.0%
catalase (Koch Light)	0.01 mg/100 ml
B vitamin solution ø	0.5% (v/v) †
B vitamins + tween 80 (TB culture grade; Honeywill-Atlas)	0.5% + 0.1%
B vitamins + tween 80 + DL methionine 74 µg/ml	0.5%, 0.1% and 7.4 µg/ml
20% trypticase (BBL)	0.5% (v/v)

* Holdeman and Moore (1972).

† as used by Varel and Bryant (1974).

ø B vitamin solution (Varel and Bryant, 1974) contained per 100 ml:
20 mg calcium D pantothenate, 20 mg thiamine hydrochloride,
20 mg nicotinamide, 20 mg riboflavin, 20 mg pyridoxine-hydrochloride,
(all BDH), 1.0 mg p-aminobenzoic acid, 0.25 mg biotin, 0.25 mg folic acid,
0.1 mg vitamin B_{12} cyanocobalamin.

The names and addresses of firms supplying materials are given in Appendix II.

were required suitable for the rapid and reliable subculture of demanding strains isolated on primary isolation plates and for use in specific procedures including fermentation testing and gas chromatographic identification of the fatty acids produced during fermentation. The general procedures of anaerobic culture, inoculum size and conditions described in Section A of the Materials and Methods were followed; incubation times varied in different experiments up to a maximum of 7 days.

Tests of a range of selected media. The growth of test strains of B. melaninogenicus ss. asaccharolyticus, Bangour nos. 2296 and 3502 and WPH no. 44, were tested in the following media: (i) PY medium, (ii) Proteose peptone-yeast extract balanced salts medium (PP3), (iii) Proteose peptone-liver infusion medium (PPLI), (iv) Proteose peptone-liver digest medium (PPLD), (v) Peptone-Lab Lemco-yeast extract medium (PLY), (vi) BM medium (BM; modified from Williams et al., 1975), (vii) Robertson's CMB, and (viii) CMB enriched with 1% Trypticase (BBL; CMB-T). Details of the formulae and methods of preparations of all media are given in Appendix I. Media nos. (i) to (iv) contained balanced salts solution (Holdeman and Moore, 1972). All cultures were checked visually after incubation for 20 h then reincubated for a further 24 h.

The influence of balanced salts on growth and pH. The growth of 5 bacteroides strains was studied in $\frac{1}{2}$PPY and in that medium enriched with 1% glucose ($\frac{1}{2}$PPYG). Four test media were prepared both with and without the balanced salts solution

of Holdeman and Moore and labelled ½PPY, ½PPYG, ½PPY-BSS and ½PPYG-BSS; 0.5% NaCl was added to the media not containing the balanced salts. The test strains included B. fragilis NCTC 9344, B. melaninogenicus ss. intermedius NCTC 9336, B. melaninogenicus ss. melaninogenicus VPI 4196, B. melaninogenicus ss. asaccharolyticus NCTC 9337 and Bangour 2296. Duplicate cultures of each test strain were incubated for each of 1, 2, 3, 4 and 7 days. Two strains, NCTC 9344 and Bangour 2296 were finally examined after 8 days. All cultures to be examined on a particular day were incubated in a single anaerobic jar. Spectrophotometric turbidity readings and the end pH of all cultures and uninoculated controls were recorded. In addition a 3-ml sample from one tube of each duplicate culture was ultrasonicated at peak noise for 10 to 15 min. until obvious clearing had occurred and the pH was recorded. The ultrasonication step was included to examine the possible effect of cell lysis in cultures of different ages on the end pH.

The effect of variation of the major ingredients in a complex medium. Williams et al. (1975) described a liquid culture medium containing 1% Trypticase (BBL), 1% Proteose peptone (Oxoid), 0.5% yeast extract (Difco), 1% glucose, 0.5% NaCl, 2% bovine serum and growth factors including haemin, menadione, cysteine hydrochloride and sodium carbonate (medium BM). This medium was modified for these growth studies by

deleting glucose and replacing bovine serum with 2% horse serum. A series of media trials were then undertaken to determine the effects of limited changes in the ingredients on the growth of strains of B. melaninogenicus ss. asaccharolyticus. The modifications tested are detailed in table 4.

The test strains included the type strain NCTC 9337, Bangour strains 2296 and 3502 and WPH strains 41, 45 and 94. Visual turbidity assessments were made after anaerobic incubation for periods up to 96 h. pH readings were taken on all complete uninoculated media and on some cultures after incubation for 44 h.

The effect of variation in the final concentration of yeast extract or Trypticase was tested in two serum-free media, nos. V and VI. Sterile 20% aqueous solutions of these two nutrients were prepared separately, presteamed, and added to the steamed and cooled basal media immediately before inoculation to give the final concentrations detailed in the table. The test strains of B. melaninogenicus ss. asaccharolyticus for this experiment were Bangour nos. 2296 and 3502 and WPH no. 44. Growth was assessed after incubation for 20 and 50 h.

Further studies with a range of demanding strains. The growth of 6 strains of B. melaninogenicus ss. asaccharolyticus was compared in 5 media. The test strains were Bangour nos. 2296 and 3502 and WPH nos. 34, 36, 37 and 44.

Table 4: Test modifications to BM medium*

Medium	Ingredients (percentage w/v or v/v)								
	Difco Tryptone	BBL Trypticase	Oxoid PP	Difco P-P No. 3	Difco Casamino acids	Difco Yeast extract	Horse serum	Meat	Water + 0.5% NaCl
I* (BM)	0	1.0	1.0	0	0	0.5	2.0	0	to 100%
II	0	1.0	0	1.0	0	0.5	2.0	0	"
III	0	1.0	0	1.0	0	0.5	0	0	"
IV	0	1.0	0	1.0	0	0.5	5.0	0	"
V †	0	1.0	0	1.0	0	0	0	0	"
VI ø	0	0	0	1.0	0	0.5	0	0	"
VII	1.0	0	0	1.0	0	0.5	0	0	"
VIII	0	0	0	1.0	3.0	0.5	0	0	"
IX	0	0	0	1.0	3.0	1.0	0	0	"
X	0	0.3	0	1.0	2.0	0.5	0	0	"
XI	0	1.0	1.0	0	0	0.5	0	+	"
XII	0	1.0	1.0	0	0	0.5	2	+	"

* modified BM medium (from Williams et al., 1975).

† yeast extract added to final concentrations of 0.25, 0.5, 1.0 and 2.0%

ø Trypticase " " " " " " " " " "

The test media included (i) medium III; (ii) medium IV, (see table 4); (iii) modified BM medium containing meat particles (BM-CM; no. XII in table 4); (iv) CMB medium, and (v) CMB medium enriched with 1% Trypticase (CMB-T). Cultures were checked visually after 20 h, reincubated and finally read after 96 h. Microscopy of wet films was performed on the 96-h cultures of WPH strains 36, 37 and 44.

Studies with vitamin B_{12}

Vitamin B_{12} (cyanocobalamin) was tested as a possible growth factor for strains of B. melaninogenicus ss. asaccharolyticus by addition to a range of enriched media. A series of media trials were carried out and the general procedures detailed earlier were followed. A number of experiments were of a more specialised nature and these are detailed below.

Organisms. The test strains of B. melaninogenicus ss. asaccharolyticus studied were the type strain NCTC 9337 and Bangour strains 2296 and 3502. A range of Bacteroides strains from other species or subspecies were included in some experiments for comparison. They were B. fragilis strains NCTC 9343 and 9344, B. thetaiotaomicron strain NCTC 10582, B. vulgatus strain NCTC 10583, B. melaninogenicus ss. melaninogenicus strain VPI 4196, B. oralis strain ATCC 15930 (but see Holbrook and Duerden, 1974), and B. melaninogenicus ss. intermedius strains NCTC 9336 and 9338.

Media, supplements and inocula. The following media were used in these experiments: (i) $\frac{1}{2}$PPY, (ii) $\frac{1}{2}$PPYS, (iii) PPY,

(iv) PPYS, (v) modified BM medium (BM; no. 1 in table 4), (vi) BM without serum (BM-S).

The inoculum used for all cultures was 0.02 ml of a 24-h BM-CM broth culture.

Vitamin B_{12} was added to the test media when required from a filter-sterilised aqueous 1×10^{-3}% (w/v) solution of cyanocobalamin (BDH). The solution was stored at 4°C protected from light. The final concentration of the vitamin in the test media was 1×10^{-5}% (1×10^{5} pg/ml). A final concentration of 1×10^{-3}% was used in a few preliminary experiments and the stock solution in these trials contained cyanocobalamin 0.1 g/100 ml. DL-methionine (DL-met; BDH) was added to ½PPY and PPY media in one experiment. Three aqueous, filter-sterilised stock solutions were prepared containing 3.7×10^{4}, 3.7×10^{3} and 3.7×10^{2} µg of the amino acid per ml. 0.2 ml of each was added to 10 ml of the basal media to give final concentrations of 740, 74 and 7.4 µg/ml respectively.

Effect of variation in the final concentration of vitamin B_{12}. A range of concentrations of vitamin B_{12} was used in two experiments designed to show the effect of changes in the vitamin concentration on the growth of test strains.

1. The test strains were B. melaninogenicus ss. asaccharolyticus, Bangour strains 2296 and 3502; B. melaninogenicus ss. melaninogenicus strain VPI 4196; ss. intermedius strains NCTC 9336 and 9338, and B. oralis strain ATCC 15930. The

strains were cultured in $\frac{1}{2}$PPY medium containing vitamin B_{12}. All strains were tested in triplicate at each of 7 different concentrations of vitamin B_{12}. The period of incubation varied for each strain and was selected to ensure good growth in the basal medium. The stock solution of vitamin B_{12} contained 0.1 g/100 ml and serial logarithmic dilutions of this solution were prepared. 0.1 ml of each dilution and the full-strength solution were added to 10-ml quantities of the basal medium giving a range of final concentrations in the test media of 1×10^7 to 10.0 pg/ml. After anaerobic incubation the turbidity of all cultures was read spectrophotometrically and the results were collated.

2. Vitamin B_{12} was added to tubes of basal PPY medium to give final concentrations ranging from 1×10^7 to 1×10^4 pg/ml. All tubes, including 2 tubes of the basal medium, were inoculated with the test strain of B. melaninogenicus ss. asaccharolyticus, Bangour 2296. Sufficient tubes were inoculated of each concentration, and of PPY medium alone, to allow incubation of separate duplicate cultures for each of 4, 8, 16, 20, 24, 30, 40, 44 and 48 h. After incubation, spectrophotometric turbidity readings were performed on all cultures. Growth was stopped by the addition of 1 drop of 40% formalin and all tubes were then stored at 4°C. Total cell counts were performed on one tube from each of the 48 h cultures in basal PPY and PPY medium containing 1×10^5 pg/ml of the vitamin.

Vitamin B_{12} studies: ^{57}Co-B_{12} uptake

The uptake of vitamin B_{12} (cyanocobalamin) by three strains of B. melaninogenicus ss. asaccharolyticus was demonstrated by scintillation counts of the supernates obtained after growth of the strains in a medium containing ^{57}Cobalt-labelled B_{12}.

Organisms. The strains studied included the type culture of B. melaninogenicus ss. asaccharolyticus NCTC 9337 and 2 clinical isolates Bangour nos. 2296 and 3502.

Preparation and labelling of the test medium. 200 ml of basal PPY medium (see Appendix I) was prepared; before autoclaving, three 10-ml quantities were dispensed into each of three 27-ml screw-capped universal containers, while the remaining 170 ml was supplemented with vitamin B_{12} as follows: 0.2 ml of ^{57}Co-B_{12} (2.0 u. Curies containing 11 ng of B_{12}) and 1.0 ml of 'cold' vitamin B_{12} containing 160 ng of B_{12}, gave a total volume of 171.2 ml containing 171 ng of the vitamin. The complete medium was dispensed in 10-ml quantities into universal containers and all containers were sterilised by autoclaving. Each 10-ml volume of labelled medium contained 10 ng of ^{57}Co-B_{12}. The 3 unlabelled tubes were prepared as comparative controls for the demonstration of growth differences that might be induced by the addition of the vitamin. All test media were enriched with the heat-labile culture supplements detailed in section A.

Inoculum. The test strains were recovered from freeze-dried ampoules in 10 ml of Robertson's CMB, subcultured into PPY medium and incubated for 2 days at 37°C. 0.02 ml of the 48-h PPY medium culture was used to inoculate the presteamed test media of strains NCTC 9337 and Bangour 2296. Bangour strain no. 3502 did not grow in the PPY medium and a 4-day CMB broth culture was used as the starter culture for this strain.

Method. The uptake of labelled B_{12} by each strain was tested on cultures incubated for 4, 24 and 48 h. The cultures at each time were incubated in separate anaerobic jars. The 4-h cultures were incubated in a 37°C water bath to minimise the warm-up time. Only one culture of Bangour 2296 was tested after incubation for 24 h and similarly only one culture of Bangour 3502 was tested at 48 h. With these exceptions all the strains were tested in duplicate at the times stated. The single uninoculated universal containing ^{57}Co-B_{12} was also incubated for 48 h.

Each of the test strains was inoculated into one of the unlabelled tubes of PPY medium and incubated for 48 h. The growth in these cultures was compared visually with the growth obtained in the 48-h vitamin B_{12}-containing cultures of each strain.

After incubation, all the ^{57}Co-B_{12} labelled cultures were centrifuged at 1000 g for 1 h in an MSE Major centrifuge. 5 ml of supernate from each culture was removed aseptically into clean, sterile universals. The remaining medium and

cells were discarded.

Scintillation counts were performed on the uninoculated control and all supernates.

Calculation of results. The uninoculated control contained 100% of the label (10 ng of vitamin B_{12}). The percentage loss of the label from the samples was calculated by comparing the counts/second (cps) obtained from the supernate alone with that obtained from the control.

Percentage recovery. The percentage recovery for this technique was calculated in a separate experiment. The three strains were cultured in triplicate in the same medium under the same conditions for 48 h and the cells deposited as before. An uninoculated control was also included. The cells were retained and washed once in physiological saline at 1000 g for 24 h in the MSE centrifuge. Scintillation counts on the resuspended cells, the saline wash and on the supernates were correlated to calculate the percentage recovery for each culture.

The addition of the labelled vitamin during preparation and the scintillation counts were kindly performed by Mr R. Samson and his staff in the department of Therapeutics, Royal Infirmary, Edinburgh.

Bulk culture in extremely anaerobic enriched media

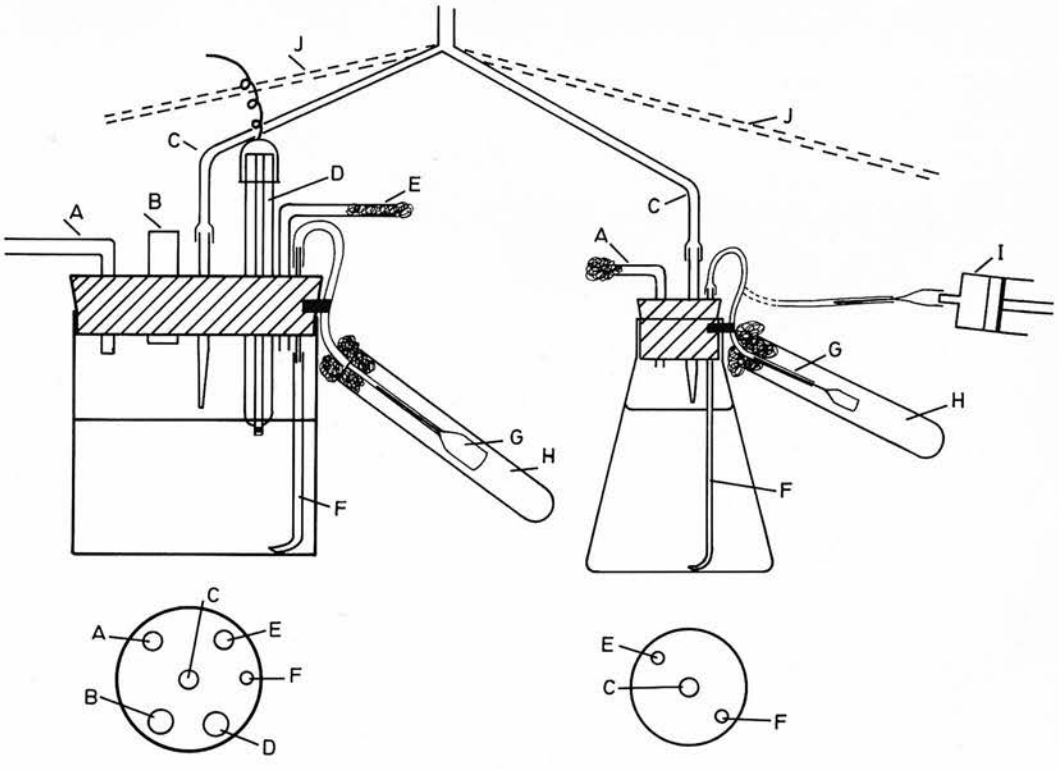
Techniques were developed to enable intermittent sampling during the continuous anaerobic growth of an asaccharolytic strain of B. melaninogenicus to compare its growth rate in

200-ml quantities of 3 different enriched broths. Stable physical and oxidation-reduction conditions were maintained by placing the culture flasks in a 37°C water bath and using the anaerobic cabinet gas mixture to produce extremely reduced cultures.

Test organism. B. melaninogenicus ss. asaccharolyticus strain no. Bangour 2296 was isolated from a wound swab taken from a patient in the Bangour General Hospital, West Lothian. It was identified in the laboratory by the procedures of Duerden et al. (1976).

Setting up the culture apparatus. Four culture vessels were used; the anaerobic cabinet exhaust gas monitor (vessel A) and three 250-ml Erhlenmeyer flasks (vessels B, C and D). The apparatus is shown in fig. 3. The anaerobic cabinet monitor was modified to provide a separate inoculation and sampling tube. The rubber bung was pierced with a headless 21 g x 1½-in. needle and 1.0 mm polyvinyl tubing was attached to both ends. The inner end was c. 7.0 cm long and extended to the bottom of the vessel. The outer end was of similar length and was strapped to the outside of the vessel. A 21 g x 1½-in. disposable needle was inserted in the free end. To minimise the risk of contamination a sterile 3 x 3/8 in. tube enclosed the needle and was held in place by a cotton wool plug. Similar inoculation and sampling tubes were fitted to the Erhlenmeyer flasks. Each of the flasks

Figure 3. A diagrammatic representation of the apparatus used for bulk culture of the B. melaninogenicus ss. asaccharolyticus strain Bangour 2296 in four sealed culture vessels while held under extremely reduced conditions by continuous flushing with an oxygen-free gas mixture containing 10% CO_2.



The vessel shown on the left is the monitoring vessel for the anaerobic cabinet exhaust gases modified to provide a separate inoculation and sampling tube. One of three 250-ml Erhlenmeyer culture flasks is shown on the right.

Key
- A = bridge connection to reservoir and the calomel reference electrode (not shown).
- B = site for pH electrode.
- C = gas inlet.
- D = platinum electrode.
- E = gas outlet.
- F = sampling tube connected to syringe needle G.
- H = protective hood for sampling connection.
- I = syringe for sampling or inoculation.
- J = gas lines to other culture vessels not shown.

Details of the media, setting up and operation are given in the text.

also carried separate 6.0-mm i.d. gas inlet and outlet tubes. The latter was bent at 90° and plugged with cotton wool. The pH electrode was omitted from the vessel A for this experiment. The preparation and sterilisation of this vessel was described in a previous section. At autoclaving, each vessel contained 200 ml of basal medium without heat-labile supplements. Vessels A and D contained BM medium (medium I, table 4); vessel B contained $\frac{1}{2}$PPY medium and vessel C $\frac{1}{2}PPY_{12}$ medium (formulae given in appendix I). Prior to sterilisation, the bungs of each vessel were taped and placed loosely in position and aluminium foil covers were fitted to maintain sterility. After autoclaving, the media were cooled to about 45°C and placed in a 37°C water bath; the bungs were pressed down, tightly sealed with wax, and the sterile polished Pt electrode was inserted aseptically before gassing commenced. The flow rate of the anaerobic cabinet gas mixture was $N_2 + H_2$ at 0.5 litres/min. and CO_2 at 50 ml/min. The tapered gas inlet of each flask was adjusted to 4.0 mm above the medium. After O_2-free gassing for 2 h, the heat-labile culture supplements used in all anaerobic media and horse serum to a final concentration of 2% were added aseptically as a single 14-ml dose through the inoculation tube. Vitamin B_{12} solution was pooled with the other additives for vessel C. The concentrations of all culture supplements added to media in this study are given in Appendix I.

After addition of heat-labile components, the completed media were left gassing overnight to provide a sterility check and ensure complete reduction prior to inoculation.

Inoculum. The inoculum used for each culture was 0.5 ml of a 20-h culture of the test strain in BM-CM medium.

Measurements taken. The cultures were not shaken during growth but were gently mixed for 20 s prior to withdrawal of 2.0 ml samples for pH and spectrophotometric turbidity readings. Samples were taken at 3-hourly intervals for the first 12 h, sampled again after 14 h and then at 24, 27, 30 and 33 h. pH readings were taken on freshly withdrawn samples and turbidity readings were made in 1.0-cm cuvettes at 600 nm as described previously.

The first Eh reading (E_{Pt}) was taken 180 s after the basal media were placed in the water bath and subsequent readings were taken at frequent intervals throughout the experiment.

SECTION D

GAS CHROMATOGRAPHY (GC) IN THE IDENTIFICATION OF THE BACTEROIDACEAE

Organisms

The sources and identity of 15 type culture or reference strains, 17 referred strains and 153 strains of Gram-negative anaerobic bacilli are summarised in table 5. In all, 185 isolates were examined.

GC media

The general procedures of media preparation and the details of all culture supplements are described in section A of the Materials and Methods section. Seven media were used for the GC studies: (i) BM medium (modified from Williams et al., 1975), (ii) full-strength PPY medium, (iii) PPY with 1% glucose (PPYG), (iv) PPY with glucose and 2% horse serum (PPYSG), (v) PPY with serum alone, (vi) half-strength PPY medium ($\frac{1}{2}$PPY), (vii) $\frac{1}{2}$PPY supplemented with vitamin B_{12} (cyanocobalamin), and (viii) $\frac{1}{2}$PPY with vitamin B_{12} and serum. For detailed formulae see Appendix I.

Instrumentation: Setting up and calibration of gas flows

A Pye Unicam (Cambridge, England) series 104 dual flame ionisation gas chromatograph, fitted with heated injection ports, was used with a Servoscribe 1S recorder, model 541.20 (Belmont Instruments, Glasgow), set at the 10 mV range with a chart speed of 120 mm per h. Cylinders of the oxygen-free nitrogen carrier gas, hydrogen and air were obtained from the British Oxygen Co, Ltd. Initial setting up and testing of the equipment was carried out by a Pye Unicam service

Table 5 : Sources of the 185 strains examined by gas chromatography of their fatty acid metabolic products

Strain	Range	Source
Bacteroides fragilis	NCTC 9344	National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale, London.
	NCTC 8560	
B. thetaiotaomicron	NCTC 10582	NCTC
	NCTC 8492	Dr Ella M. Barnes, Agricultural Research Council, Food Research Institute, Norwich NOR 7OF.
B. distasonis	NCTC 8503	Dr Barnes
B. ovatus	NCTC 8483	Dr Barnes
B. vulgatus	NCTC 8482	Dr Barnes
	NCTC 10583	NCTC
B. melaninogenicus ss. intermedius	NCTC 9336	NCTC
B. melaninogenicus ss. asaccharolyticus	NCTC 9337	NCTC
Fusobacterium polymorphum	NCTC 10562	NCTC
F. necrogenes	NCTC 10723	NCTC
F. necrophorum	NCTC 10575	NCTC
Leptotrichia buccalis	NCTC 10249	NCTC
B. oralis (B. melaninogenicus, see Holbrook and Duerden, 1974)	ATCC 15930	American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A.

Table 5 CONTD.

The following strains were referred by the International Committee on Systematic Bacteriology (I.C.S.B.) Taxonomic Subcommittee for Gram-negative rods.

Strain	Range	Source
B. melaninogenicus ss. melaninogenicus	WAL 2721	Dr S.M. Finegold, UCLA School
	WAL 2724	of Medicine, Los Angeles,
		Ca 90024, U.S.A.
B. melaninogenicus ss. melaninogenicus	GUI 1011	Dr K. Ueno, Department of
	GUI 1034	Bacteriology, Gifu University
		School of Medicine, Tsukasa-Machi,
		Gifu-shi, Gifu-ken, Japan
	VPI 4196	Dr Elisabeth Cato, Virginia
		Polytechnic Institute and State
		University (VPI), Blacksburg Va.
		24060, U.S.A.
B. oralis	VPI 7570A,	VPI
	VPI 5832	VPI
	J1, 7CM, 30	Dr B.A. Phillips, National
		Institute for Research in
		Dairying, University of Reading,
		England.
	NP 333	Mr G.H. Bowden, London Hospital
		Medical College, Dental School,
		Turner Street, London, England.
B. ochraceus (Ristella ochraceus)	1956C, 2467B	Dr Madelaine Sebald, Anaerobic
		Laboratory Institut Pasteur,
		25 Rue du Docteur Roux,
		Paris XV^{e}, France.
	VPI 2845	VPI
	10, 79B, 73	Dr W.H. van Palenstein-Helderman,
		Department of Preventive Dentistry,
		University of Utrecht, Netherlands.

Table 5 CONTD.

A further 113 strains of Gram-negative anaerobic bacilli were isolated from subgingival dental plaque, 40 strains from clinical specimens (19 from faeces, 19 from high vaginal swabs, 1 rectal abscess and 1 abdominal wound swab).

engineer. Preparation of the instrument for analyses and the calibration of gas flows was performed by me and followed the recommendations in the Pye Unicam instruction manual.

Column packing. Two identical coiled glass columns (Pye Unicam, 5 m x 4 mm i.d.) were packed in the laboratory with Chromosorb 101 (80/100 mesh; Johns-Manville, Denver, Colo; supplied by Gas Chromatography Services Ltd).

Small amounts of the polymer were added through a funnel attached to the open end, and gentle water pump suction was applied to the effluent end. The effluent end was plugged with a small amount of glass wool. Gentle rotation of the coil and occasional tapping with a rubber bung fixed into the end of a 6" length of cardboard tube was used to assist packing, but prolonged agitation or vibration was avoided. On completion of packing the open end was plugged with a small amount of silane-treated thread (see Horvath, 1975; available from Pye Unicam) kindly supplied by Miss Margaret Lawson (technician to Professor G.S. Boyd) in the department of Biochemistry. The columns were then conditioned for 24 h at 250°C in the chromatograph oven. During this operation the effluent end was not attached to the detector inlets but remained free. The detector inlets were sealed off as recommended in the instruction manual. After conditioning and cooling the columns were gently repacked using suction and a minimum of tapping; fresh polymer was added to leave

a 2.5 cm gap at the top of the column. After further overnight conditioning at 250°C, the columns were purged at the operating temperature of 190°C for 16 h before use.

Operating conditions. The chromatographic conditions for analysis of fatty acid products are summarised in table 6. The carrier gas and hydrogen flow rates of the non-analysing column were adjusted for optimum acetic acid response. The columns were kept in condition by maintaining the flow of carrier gas overnight at the operating temperature. There were no problems of column bleed with the porous polymer. Flow rates were checked each day before analyses commenced.

Sample preparation and analysis. The procedures were derived from those of Carlsson (1973). In general, GC analyses were performed on two-day cultures; four- or seven-day cultures were used for a few slow-growing strains. Cultures were acidified with 4 drops of 50% H_2SO_4 to approximately pH 2.0 and centrifuged at 800 *g* for 1 h; the cell-free supernates were stored at 4°C in screw-capped 7-ml bijou bottles until analysis. For analysis of volatile products, a 0.6-µl sample of supernate was injected directly on to the analysing column with a 5.0-µl syringe (SGE, Melbourne, Australia, available from Phase Separations Ltd). No pretreatment or extraction procedures were used. Contamination of the top 2-3 cm of the column occurred during volatile acid analyses and necessitated periodic replacement with fresh

Table 6: Operating conditions for gas chromatography

Instrument: Pye-Unicam series 104 and a Servoscribe series 541.20 recorder

Isothermal operation only		Backing off range	: X 1
Injection point heater	: 190°C	Attenuation setting vfa*	: 2×10^2
Column oven temperature	: 190°C	nvfa	: 5×10^2
Detector oven "	: 250°C	Recorder speed	: 120 mm/h
Carrier gas (N_2) flowrate	: c. 35-ml/min	Injection volume (both analyses)	: 0.6 µl
Hydrogen " "	: " 35 "		
Air "	: " 600 "		

* vfa = volatile fatty acids; nvfa = non-volatile (methylated) fatty acids

polymer. Overnight conditioning following by purging for 8 h restored normal operation.

For non-volatile acids the acidified culture supernates were methylated according to the method of Holdeman and Moore (1972) and a 0.6-µl sample of the chloroform extract was injected on to the column operated under the same conditions used for volatile analyses.

Identification of fatty acids in test samples. The chromatographically separated peaks were routinely identified by direct measurement of the relative retention times, with the acetic acid peak as a reference. Accurate identification was made by comparing retention times of test peaks with those obtained from a series of chromatographically pure aqueous standards.

Standards. Single aqueous 0.01M standards of acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids (BDH Analar) were used in the volatile analyses to establish absolute retention times and quantitation values. A combined acid standard containing 0.01M of each acid was used to establish relative retention times; it was used each day to monitor column performance and sensitivity settings of the instrument. Aqueous 0.02M lactic and succinic acid standards were included with each batch of non-volatile (methylated) acid analyses. Samples of uninoculated sterile medium were included as controls in every batch of each type of analysis.

Quantitation. The approximate concentration values of acids (µmols per ml) in the test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. The calculation used was as follows:

If peak height is proportional to the concentration of the acid in the sample then:

$$\text{Concentration of acid (µmols/ml)} = \frac{A - B}{C} \times S$$

where A Height in mm of test peak

B " " " " peak for test component present in uninoculated medium

C Height in mm of appropriate acid standard

S Concentration of acid standard (µmols/ml)

N.B. 0.01M = 10 µmols/ml

SECTION E

IDENTIFICATION AND CHARACTERISATION OF BACTEROIDACEAE

The characterisation scheme developed by Duerden, Holbrook, Collee and Watt (1976) was used to identify all the strains examined in these studies. Table 7 lists the complete range of tests used to identify the strains. The methods and appropriate controls used have been detailed by Duerden et al. (1976) and by Holbrook (1976). A number of modifications or additions to the range of tests have been subsequently introduced to allow identification of a wider range of isolates or to improve the discrimination of certain subgroups already examined according to the scheme.

Additional tests or modifications to existing tests

Gas chromatography. The GC procedures developed for analysis of the fatty acid metabolic products of bacteroides strains are detailed in Section D of the Materials and Methods. A total of 185 strains were studied and included 40 strains examined as part of a collaborative investigation of the B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus group instigated by the I.C.S.B. Taxonomic Sub-committee for Gram-negative anaerobic rods. A further 15 strains referred by interested workers have subsequently been characterised. The strains, listed in table 8, include the reference strain for the proposed new subspecies B. melaninogenicus ss. levii, VPI 3300. This strain, obtained by the author from the VPI anaerobe laboratory (Dr Lillian V. Holdeman) was previously

Table 7: Tests for the characterisation of Bacteroidaceae*

(after Duerden et al., 1976)

Colonial morphology	Catalase production
Cellular morphology	H_2S production
Haemolysis on blood agar	Nitrate reduction†
Pigment production on lysed blood agar	Indole production
Motility	Gelatinase activity
Lipase activity	Aesculin hydrolysis
Oxidase production	Dextran hydrolysis

Carbohydrate fermentation tests with the following sugars:

Glucose	Maltose	Trehalose
Lactose	Rhamnose	Mannitol
Sucrose		

Tolerance tests: a) Bile salts

0.5% sodium taurocholate (BDH) 0.1% sodium deoxycholate (BDH)

0.5% sod. taurocholate + 0.1% sod. deoxycholate

b) Dyes$^{\emptyset}$

Brilliant green	1/80,000
Gentian violet	1/100,000
Victoria blue 4R	1/80,000
Ethyl violet	1/80,000

Table 7 CONTD.

Antibiotic resistance disk tests with the following antibiotics:§

neomycin sulphate 1000 µg	kanamycin sulphate 1000 µg
benzyl penicillin 1.5 units	erythromycin ethyl succinate 60 µg
colistin sulphate 10 µg	rifampicin 15 µg
vancomycin 15 µg	chloramphenicol 10 µg
methicillin 10 µg	lincomycin 2 µg
clindamycin 2 µg	bacitracin 0.1 unit
metronidazole 5 µg	kanamycin 30 µg

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* see Duerden et al. (1976) and Holbrook (1976) for details of media and methods

† Nitrate reduction medium now modified (q.v.)

ø All dyes obtained from Geo. T. Gurr and sons Ltd.

§ Antibiotic disks were prepared in the laboratory or obtained from Mast Labs Ltd.

Table 8 : The source and identity of additional referred strains of *Bacteroides* spp. characterised in this study

Strain No.	Species or subspecies	Source*
NCTC 10825	*B. splanchnicus*	NCTC
NCTC 10826	*B. splanchnicus*	NCTC
VPI 8906	*B. oralis*	Dr Ella M. Barnes
VPI 9958	" "	" " " "
5540	" "	" " " "
7880	" "	" " " "
WAL 3030	" "	Dr S.M. Finegold, Wadsworth Anaerobic Laboratory
WAL 3281	" "	Dr S.M. Finegold
VPI 8057	*B. disiens*	Dr Barnes
VPI 7852	" "	" "
VPI 6822	*B. bivius*	" "
VPI 6318	" "	" "
GA 33	*B. ruminicola* ss. *brevis*	" "
VPI 3300	*B. melaninogenicus* ss. *levii*	Dr Lillian Holdeman V.P.I.
Gnab 55s	*Bacteroides* sp. (Wound swab)	Dr R. Miles, R.I.E.

* For addresses, see Appendix II.

designated B. melaninogenicus, Lev group 1, and was originally isolated from the rumen (Lev, 1958; Lev, Kendell and Milford, 1971). All the additional strains were characterised in the full scheme including GC analysis and, in conjunction with appropriate control strains, were used to evaluate the additional tests described below.

Nitrate reduction. In the development of the characterisation scheme Duerden et al. (1976) attempted to restrict all routine biochemical testing, in liquid media, to two media - a thioglycollate medium and Robertson's CMB; the use of thioglycollate medium for nitrate reduction tests presented difficulties with positive control strains producing inconsistent results. This test was not considered essential for identification of the strains originally studied and was therefore not pursued at that time (Duerden, personal communication). In setting out to characterise a wider range of clinical isolates it was decided to test the Indole-Nitrite medium (BBL) used by Holdeman and Moore (1972) and others. The presence of nitrite ions was indicated by a deep red colour when 0.5 ml of Nitrate solution A and 0.5 ml of Nitrate solution B (Litchfield, 1967) were added to a 48-h culture. Negative results were confirmed by the addition of a knife point of zinc dust to the culture. The development of a deep red colour within 1 minute of adding the zinc dust indicated that the test reagents were working satisfactorily. Reagent B contains 10 g of N-(1-naphthyl)ethylene-diamine dihydrochloride in distilled water. This substance is known to be carcinogenic and extreme precautions were taken

during its preparation and use. The control strains detailed by Duerden et al. (1976) were used in these experiments.

Bile stimulation. The bile salt tolerance tests developed by Duerden et al., were supplemented by testing for stimulation of growth by 20% ox bile (2% oxgall, Oxoid) in PYG medium (Holdeman and Moore, 1972). The effect of replacing PYG medium with PPYG medium was also tested with the strains listed in table 8 . Two reference strains, B. fragilis NCTC 9344 and B. melaninogenicus ss. intermedius NCTC 9338, were included in all trials as positive and negative controls respectively.

Fermentation testing. The sugars arabinose and xylose were added to the list of fermentable carbohydrates tested. The fermentation of these sugars has been suggested to allow the discrimination of B. ruminicola from B. oralis strains (Holdeman and Moore, 1972; personal communication, Dr Lillian V. Holdeman, 1977).

Problems in fermentation testing examined with selected strains of B. melaninogenicus

Problems encountered in the routine fermentation testing of some clinical isolates led to a comparison of glucose fermentation in 3 different media using a range of clinical isolates and reference strains representing different subspecies of B. melaninogenicus. The basal media enriched with 1%

glucose were (i) Robertson's CMB, (ii) ½PPY and (iii) the thioglycollate medium already in use. The growth of all test strains was assessed in each medium. Maltose fermentation was re-tested in thioglycollate medium alone. The original characterisations included GC analyses on 2 or 7 day cultures in PPYSG medium and pH readings and growth levels had been recorded on these cultures at the completion of the incubation period. All the test strains were also subjected to glucose utilisation studies on the ½PPY and ½PPYG cultures.

Glucose utilisation studies

½PPY medium was the basal medium for all strains. It was prepared as a single 4000-ml batch, divided into two 2000-ml aliquots and autoclaved without added growth factors. The growth supplements detailed earlier in Section A of the Materials and Methods were added to the autoclaved and cooled medium to the previously stated concentrations. 20% glucose (½PPYG) was added to one half to a final concentration of 1%. Physiological saline was added to the other half (½PPY) to the same final concentration. The completed media were dispensed aseptically in 10-ml quantities into 5 x 5/8-in test tubes fitted with loose fitting caps and stored anaerobically at 37°C for 48 h before use to reduce the media and check for any contamination. These procedures were designed to minimise losses during autoclaving and to ensure that all cultures contained the same final concentrations of nutrients.

Organisms. The strains studied are listed in table 9. They include 5 type culture or reference strains and 13 strains freshly isolated from clinical material.

Culture inocula. The test media were inoculated with 0.02-ml quantities of a 24-h culture in BM-CM medium. It was found necessary to change the starter culture when some of the clinical isolates showed poor growth in Robertson's CMB medium after incubation for 24 h.

Method. Four sets of duplicate tubes of $\frac{1}{2}$PPY and $\frac{1}{2}$PPYG media were inoculated for each test strain. Each set of tubes was placed in a separate anaerobic jar along with one set of uninoculated tubes per jar and incubated anaerobically. One jar was removed on each of the first 2 days, on the fourth day and finally after incubation for 7 days. The growth in all incubated cultures was resuspended, mixed well and divided into 3 x 3-ml samples. Spectrophotometric turbidity readings were performed on one sample with an uninoculated control as a reference. pH readings were taken on the second sample after preparation of Gram-stained smears for purity checks. The third sample was centrifuged at 800 g for 30 min at room temperature and the supernate was collected. The supernate was diluted 1 in 4 with sterile distilled water and a 50-µl sample used to estimate true glucose levels.

Glucose estimations. True glucose levels were assayed by the glucose oxidase method of Morin and Prox (1973).

Table 9 : Strains of B. melaninogenicus tested in the glucose utilisation studies

Strain	Identity
NCTC 9337	B. melaninogenicus ss. asaccharolyticus
NCTC 9336	" " ss. intermedius
VPI 4196	" " ss. melaninogenicus
ATCC 15930	B. oralis (B. melaninogenicus ss. melaninogenicus)
VPI 3300	B. melaninogenicus ss. levii
WPH 57	B. melaninogenicus ss. asaccharolyticus
98	" " " "
118	" " " "
201	" " ss. N.D. ? levii
202	" " ss. intermedius
210	" " " "
214	" " " N.D. ? levii
222	" " " N.D. ? levii ? asaccharolyticus
223	" " " N.D. ? levii ? asaccharolyticus
226	" " " N.D. ? levii ? asaccharolyticus
228	" " " N.D. ? intermedius
229	" " " N.D. ? intermedius
234	" " " N.D. ? levii ? asaccharolyticus

N.D. Subspecies not definite (see results).

Duplicate tubes of uninoculated medium, also diluted 1 in 4, were included with each batch of test samples assayed. A volumetrically prepared aqueous standard containing 1% glucose was also diluted 1 in 4 and assayed at the same time. For testing, 2.5 ml of the single test reagent was mixed in a 3 x 3/8 test tube with 50 µl of sample and left to stand for 2 to 5 min. before readings were made against a reagent blank, in 1.0-cm matched cuvettes, in a Pye Unicam SP600 spectrophotometer set at 465 nm.

Calculation of results. The percentage of glucose utilised by each test strain, at each incubation time, was calculated by comparing the mean absorbance value for each pair of duplicate test samples with the mean absorbance value of the duplicate samples of uninoculated medium containing 100% of the glucose test substrate.

RESULTS

Handling procedures and redox potentials

When the present author joined the Edinburgh team it had been demonstrated that improvements to bench procedures for anaerobic culture (Collee et al., 1972), improvements to culture media (Finegold, Sugihara and Sutter, 1971; Gibbons and MacDonald, 1960) and the incubation of all anaerobic cultures in 10% CO_2 (Watt, 1973) made possible the quantitative recovery of clinically important strains of the Bacteroidaceae on solid media and provided good growth in liquid media (Duerden et al., 1976). A limited number of additional experiments were planned to confirm and extend the earlier findings.

Presteaming media prior to inoculation

Steaming Robertson's CMB medium for 30 min. caused a significant fall in the redox potential of the uninoculated medium. Six broths, equilibrated in air overnight, all had similar, stable E_{Pt} values of + 130 mV ($\pm$ 5) both in the broth above the meat layer and in the meat layer itself. The pH of the broths was 6.8 $\pm$ 0.05. Thus if the potential of the saturated KCl calomel electrode relative to a standard hydrogen electrode is + 241 mV at 25°C and pH 7.0 (Jacob, 1970), the Eh of the broths was approximately + 370 mV. After steaming for 30 min., the broths had reached E_{Pt} values ranging from -70 to -110 mV

or Eh values of + 170 to + 130 mV. Readings taken within the meat layer of 3 steamed broths were uniformly lower than the readings taken above the meat particles. The mean Eh in the meat layer was + 80 mV. In one steamed broth for which the Eh in the broth was + 135 mV and + 70 mV in the meat layer, the electrode and the salt-agar bridge were left in place after the initial readings and further readings taken 1 h later. A slow negative drift occurred and the Eh above the meat was then + 70 mV and in the meat layer it was -100 mV.

The measuring electrode was found to adjust quickly to the redox potential of the test media after transfer from either the holding broth or the previous sample. The major adjustment occurred in less than 30 s and was followed by a slow drift that was virtually complete within $1\frac{1}{2}$ - 2 min. Readings were taken at 180 s. If the electrode was pushed into the meat particles after the initial reading the negative change was clearly evident.

Continuous monitoring of anaerobic cabinet exhaust gases

The potentiometric device was adapted to allow continuous monitoring of the exhaust gases from an anaerobic cabinet. In principle an extremely reduced condition will be developed in an uninoculated organic medium subjected to continuous gassing with an oxygen-free gas (Hungate, 1950;

Jacob, 1970). The effluent gas from an operational anaerobic cabinet, free from leaks or contamination by air trapped in equipment introduced into it, should produce and maintain such a reduced state.

In practice it was found that stable reduced conditions were generally established in Trypticase soy broth in $2\frac{1}{2}$ - 3 hours. The actual time taken varied and depended on the E_{Pt} value when gassing commenced. A stable E_{Pt} of at least -580 mV could be maintained for long periods of time. In one trial a stable E_{Pt} of -580 mV was maintained continuously for over 2 months. Reduction of the gas flow into the cabinet to a trickle rate for 2 days or more caused a small rise to an E_{Pt} of -570 mV. The original redox potential was quickly recovered when the flow rate was increased to - 0.1 litres/min. Considerable care and attention to detail in setting up the monitor was essential to achieve optimum results. However after one or two preliminary trials it was found that the equipment could be set up quickly and consistently. Of particular importance was the cleaning and polishing of the platinum electrode. The final potential (E_{Pt}) achieved was adversely affected by inadequate polishing of the electrode. Typically the final E_{Pt} achieved with a clean but unpolished electrode was between -300 and -500 mV.

Two experiments were performed to determine the magnitude and rate of response of the electrode in this system to contamination of the exhaust gases by air. Fig. 4 shows the change in Eh when the reduced medium was aerated for 30 s, and fig. 5 shows the changes in redox potential with time when an aerated medium was reduced by gassing with an oxygen-free gas mixture containing 10% CO_2, then aerated for 4 min. and finally flushed again with the O_2-free gas mixture.

In routine operation of the cabinet the redox potential remained steady and was not affected by pressure changes induced by manipulation of the gloves.

Comparison of anaerobic cabinet and standard bench procedures

In the first of two trials to compare the anaerobic cabinet and standard bench procedures, each of the 6 reference strains listed in the Materials and Methods section showed clearly visible growth after incubation for 2 days in both the PYG medium inoculated on the bench and in the PRAS/PYG medium inoculated in the cabinet. Growth after incubation for 3 days was increased in all cases. A change in inoculum size from 0.02 to 0.04 ml, made no discernable difference to the final levels of growth seen. The C. oedematiens type D strain NCTC 8350 produced micro-colonies in all cultures. The anaerobic coccus NCTC 9801 and the Leptotrichia buccalis strain NCTC 10249 produced less turbidity in the test medium

Figure 4. The change in redox potential when a reduced medium is aerated for 30 s while gassing with an oxygen-free gas mixture containing 87% N_2: 10% CO_2: 3% H_2 at a steady flow rate of 1 litre/min.

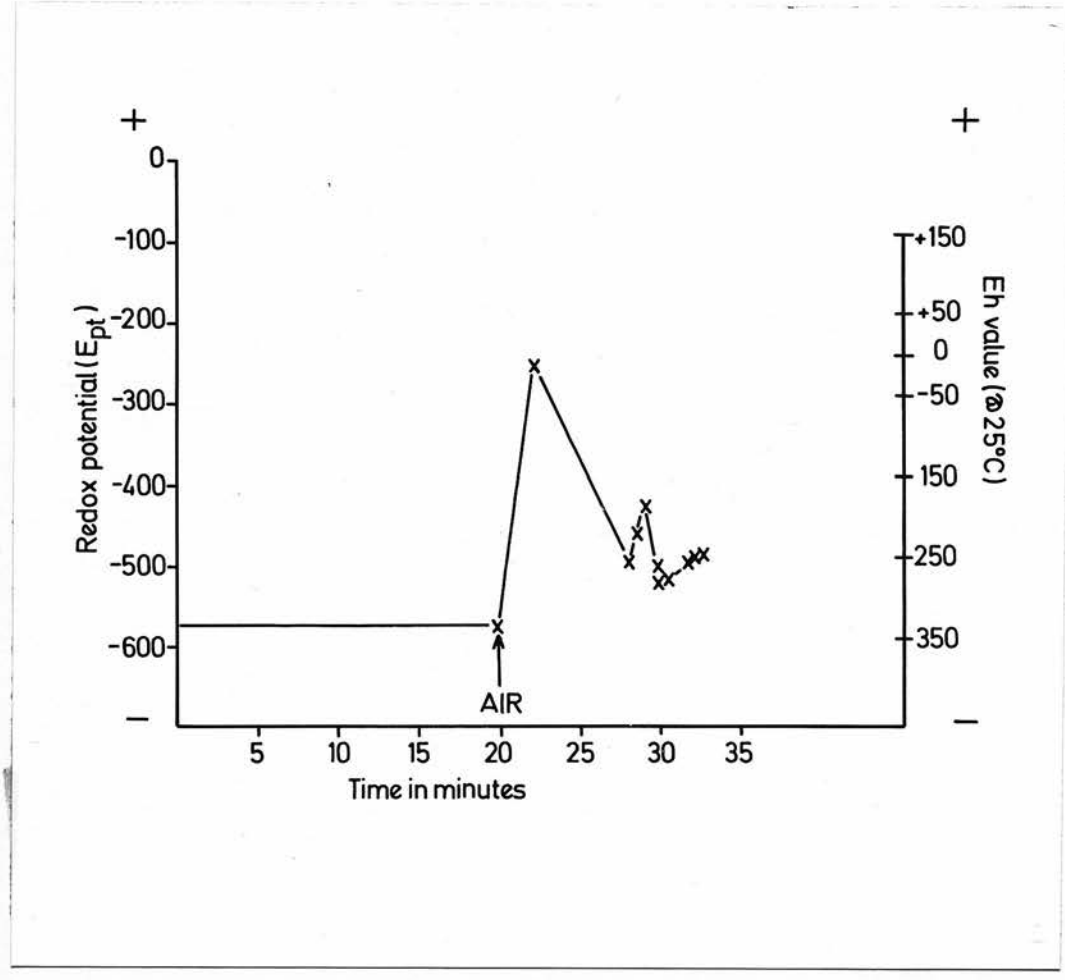
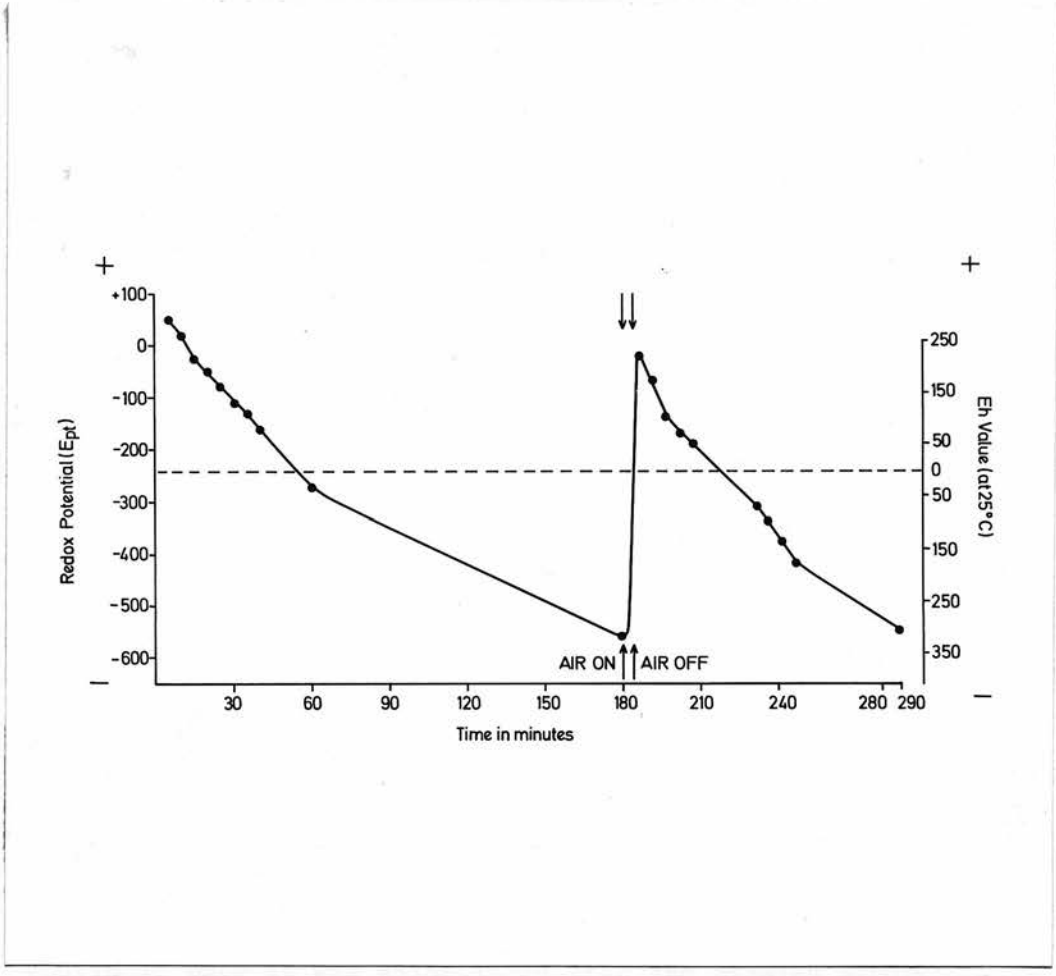


Figure 5. The changes in redox potential with time in an aerated medium subjected to a steady flow of oxygen-free gas* at 1 litre/min, re-aerated for 4 min., then re-reduced.



* 10% CO_2 was incorporated in the gas mixture. The EPt readings were not corrected for any pH changes in the uninoculated medium.

than the other strains tested and failed to reach 2+ levels after incubation for 3 days. All other test strains consistently reached 2 to 3+ levels in the same time.

Recovery and subculture of exacting strains

The type strain of B. ruminicola GA33 and the Gemmiger formicilis strains ATCC 27749 and NE3/247 were recovered pure from freeze-dried ampoules in the anaerobic cabinet. All strains were grown in VL, BM-CM and Robertson's CMB media and serially transferred through the same media inoculated both in the cabinet and on the bench using the standard anaerobic procedures of Collee et al. (1972).

In either the recovery or the amount of growth recorded in the different media there were no observable differences that could be attributed to the different methods of manipulation. Table 10 shows the density of growth, as observed by microscopy of wet films and Gram strains, achieved at each passage by each strain in the test media. Serial transfers through cabinet-inoculated media were not continued once it was established that the strains could be successfully maintained by the bench procedures. The morphological characteristics of the strains remained stable throughout the transfers. All strains grew well in the VL and BM-CM media, but growth in Robertson's CMB medium was generally less reliable and less luxuriant. Occasional growth failures were recorded; the B. ruminicola

Table 10: Density of growth* achieved by 3 obligately anaerobic Gram-negative bacterial strains during serial transfers in a range of media processed (i) in the anaerobic cabinet†, and (ii) on the bench by the standard anaerobic procedures of Collee et al. (1972)

Culture method	Test strain	Medium	Days of incubation	Amount of growth observed* at Pass no. 1	2	3	4	5
		VL	2	-	++	•••		
			4	-	+++	•••		
Cabinet	B. ruminicola GA 33	CMB	2	±	•••			
			4	±	•••			
		BM-CM	2	++	•••			
			4	++	•••			
		VL	2	++	++	++	++	±
			4	+++	+++	+++	++	+
Cabinet	Gemmiger formicilis ATCC 27749	CMB	2	-	•••			
			4	±	•••			
		BM-CM	2	++	•••			
			4	++	•••			
		VL	2	+++	+++	+++	+++	+++
			4	+++	+++	+++	+++	+++
Cabinet	Gemmiger formicilis NE3/247	CMB	2	-	•••			
			4	-	•••			
		BM-CM	2	+	•••			
			4	+	•••			
		VL	2	++	C	+++	•••	•••
			4	++	C	+++	+++	+++

Table 10 CONTD.

Culture method	Test strain	Medium	Days of incubation	Amount of growth observed* at Pass no. 1	2	3	4	5
	B. ruminicola							
Bench	GA 33	CMB	2	•••	•••	•••	•••	+++
			4	±	±	±	+++	+++
		BM-CM	2	+++	+++	+++	•••	+++
			4	+++	++	++	+++	+++
		VL	2	+++	C	++	•••	•••
			4	++	C	+++	+++	+++
	Gemmiger formicilis							
Bench	ATCC 27749	CMB	2	+	±	++	•••	+++
			4	++	C	±	+	±
		BM-CM	2	++	++	+++	•••	+++
			4	++	+	++	++	+
		VL	2	+	C	+++	•••	•••
			4	++	C	+++	+++	+++
	Gemmiger formicilis							
Bench	NE3/247	CMB	2	±	+	+++	•••	+++
			4	±	+	++	+++	++
		BM-CM	2	±	++	+++	•••	+++
			4	+	+++	+++	+++	++

* Read by microscopy of wet films and Gram stain

† See text

••• Not tested C Contaminated

strain failed to grow on one occasion after 4 days in cabinet-inoculated VL medium and the Gemmiger formicilis strain NE3/247 also failed to grow once in cabinet-inoculated CMB medium. Some difficulty was encountered in assessing the growth of the B. ruminicola strain in CMB medium inoculated on the bench. This strain stains poorly by Gram stain and was pleomorphic, ranging from small cocci to quite large rods (3-4 μm long). In CMB medium the cocci predominated and were sometimes difficult to differentiate from meat debris in the medium. Phase contrast microscopy was the most reliable means of assessment and this was confirmed by successful subculture as shown in the table.

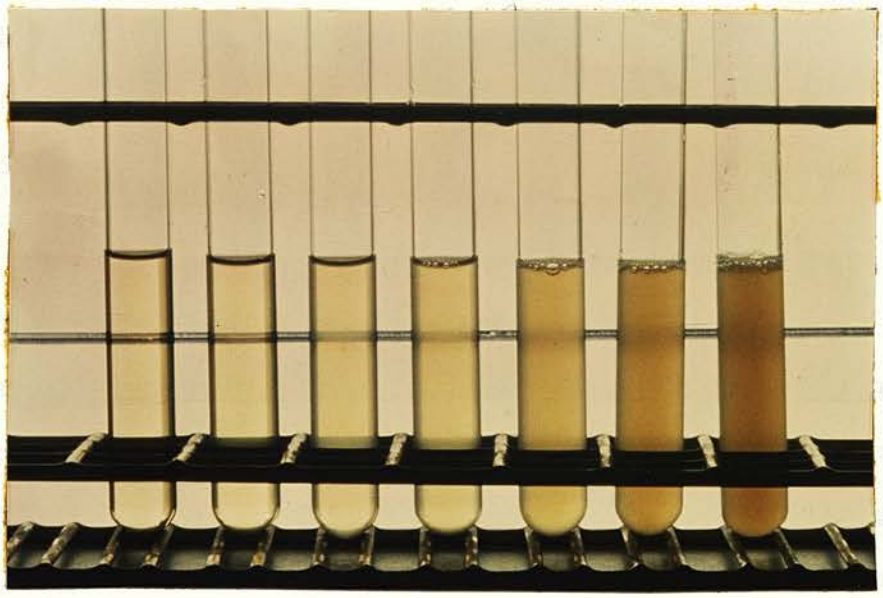
Growth studies with B. melaninogenicus ss. asaccharolyticus

Assessment of bacterial growth by culture turbidity

Plate 3 shows the 6 selected levels of turbidity recognised by visual assessment of cultures in the growth studies. A 48-h PPYSG culture of B. fragilis NCTC 9344 was used to prepare the illustration and the absorbance of each preparation at 600 nm is recorded. The absorbance of the 5+ level is a minimum figure. In one experiment, during the growth studies, independent visual and spectrophotometric readings were taken on 150 cultures. The distribution of the absorbance readings for each of the grades of turbidity

Plate III.

The six selected levels of turbidity recognised by visual assessment of cultures in the growth studies



Absorbance : 0.00 0.1 0.25 0.53 1.02 1.42 2.2

The *B. fragilis* strain NCTC 9344 was cultured for 48 h in PPYSG medium to obtain the data for this figure. The absorbance value of each tube was read at 600 nm in 1-cm cuvettes (see Materials and Methods).

is illustrated in fig. 6. The plot of the absorbance readings obtained from the 6 reference tubes is included for comparison. The figure shows that visual assessment is more reliable in the ± to 3+ range with little overlap occurring between the grades, but more difficulty was experienced in assessing cultures showing large amounts of turbidity. The maximum point of the 5+ grade is not shown but in the experiment some cultures produced absorbances of ≥ 3.0 (projected readings obtained after appropriate dilution).

Correlation of culture turbidity and total cell count

The relationship of culture absorbance to total cell numbers was evaluated with 4 bacteroides strains. Table 11 gives the total number of cells/ml of each test strain in the CMB starter cultures, the probable number of cells in the test inocula and the final number of cells/ml achieved by each strain in the 48-h PPYSG cultures. Turbidity readings taken on the doubling dilutions prepared from the PPYSG cultures were plotted in fig. 7 against the probable number of cells/ml (PNC) present in each dilution. The absorbance readings of the 6 selected grades are given for comparison.

The absorbance values of the freshly inoculated PPYSG cultures of each test strain were 0.020 or less. Similar readings were obtained on the more dilute samples of the

Figure 6. The absorbance readings of 150 cultures graded according to independent visual turbidity assessments performed on the same cultures to show the correlation of the two methods of assessing bacterial growth.

The figure shows the curve linking the absorbance readings obtained from the 6 selected visual grades determined with a 48-h PPYSG culture of B. fragilis NCTC 9344.

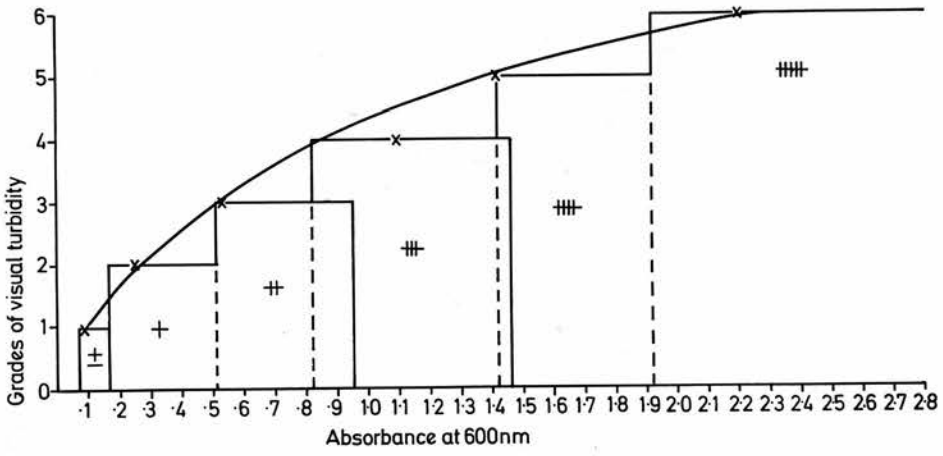
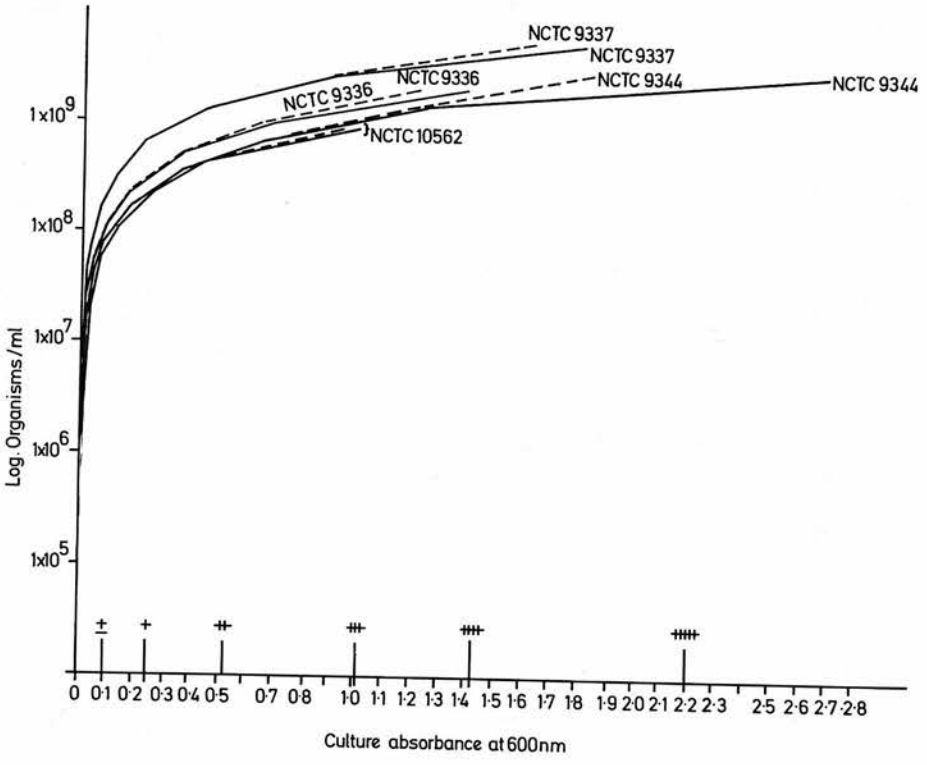


Table 11: The total number of cells/ml in 48-h cultures of 4 bacteroides strains in Robertson's CMB medium and PPYSG medium

Test strain	CMB starter: Dilution counted	CMB starter: Total no. of cells/ml	PPYSG test medium: Inoculum (PNC*)	PPYSG test medium: Dilution counted	PPYSG test medium: Total no. of cells/ml
B. fragilis BCTC 9344	1×10^2	8.6×10^9	1.72×10^7	1/128	2.75×10^9
B. melaninogenicus ss. asaccharolyticus NCTC 9337	1×10^2	7.3×10^9	1.46×10^7	1/128	5.12×10^9
B. melaninogenicus ss. intermedius NCTC 9336	1×10^2	1.49×10^9	2.98×10^6	1/128	1.95×10^9
F. polymorphum NCTC 10562	1×10^2	1.2×10^8	2.4×10^5	1/128	8.8×10^8

* PNC = the probable number of cells in a 0.02 ml inoculum.

Figure 7. The relationship of culture absorbance to total cell numbers evaluated with four bacteroides strains.



The turbidity readings were taken on doubling dilutions prepared from PPYSG cultures and the probable number of cells/ml in each dilution was calculated from an actual count on the 1 in 128 dilution of each culture.

48-h PPYSG cultures. Table 12 shows that the absorbance readings for dilutions of 128 or more of all test strains was 0.020 or less. The F. polymorphum strain reached this level at the 1 in 64 dilution. The results on these dilutions show a tendency to plateau with 3 of the 4 test strains; only the B. fragilis strain produced absorbance readings that retained the even progression noted in the less dilute samples. It was found necessary to dilute the most concentrated samples until readings below 0.500 were obtained in order to maintain a reasonable degree of correlation. There were strain-to-strain differences in the amount of turbidity produced during growth and the actual starting inoculum seemed to have an important effect on the final turbidity achieved by the test strains in the period of incubation. At one extreme the F. polymorphum strain produced the lowest maximum turbidity in 48 h but increased its cell numbers by a factor of 3500 (c. 2.4×10^5 to 8.8×10^8). By contrast the B. fragilis strain produced the highest number of cells and the highest turbidity increase but the cell numbers only increased by a factor of 150 (c. 1.72×10^7 to 2.5×10^9). These figures are consistent with the F. polymorphum strain producing about 12 generations or cell doublings in the same time that the B. fragilis strain produced 7 or 8 generations. The other test strains produced results falling between these two extremes. B. melaninogenicus ss. asaccharolyticus, NCTC 9337, increased

Table 12: The absorbance and probable number of cells/ml in serial dilutions of cultures of 4 bacteroides strains grown for 48 h in PPYSG medium

Test strain		Dilution factor 0	2	4	8	16	32	64	128	256	512
B. fragilis NCTC 9344	PNC*	2.75×10^9	1.38×10^9	6.88×10^8	3.44×10^8	1.72×10^8	8.59×10^7	4.3×10^7	2.15×10^7	1.07×10^7	5.37×10^6
	Abs	2.68 (1.84)	1.34 (1.174)	0.67 (0.654)	0.355	0.18	0.09	0.045	0.02	0.01	0.001
B. melaninogenicus ss. asaccharolyticus NCTC 9337	PNC	5.12×10^9	2.56×10^9	1.28×10^9	6.4×10^8	3.2×10^8	1.6×10^8	8.0×10^7	4.0×10^7	2.0×10^7	1.0×10^7
	Abs	1.82 (1.620)	0.91 (0.854)	0.46	0.222	0.13	0.68	0.035	0.015	0.015	0.008
B. melaninogenicus ss. intermedius NCTC 9336	PNC	1.95×10^9	9.55×10^8	4.88×10^8	2.44×10^8	1.22×10^8	6.09×10^7	3.05×10^7	1.52×10^7	7.62×10^6	3.81×10^6
	Abs	1.38 (1.222)	0.69 (0.654)	0.35	0.18	0.1	0.05	0.025	0.015	0.015	0.015
F. polymorphum NCTC 10562	PNC	8.8×10^8	4.4×10^8	2.2×10^8	1.1×10^8	5.5×10^7	2.75×10^7	1.38×10^7	6.88×10^6	3.44×10^6	1.72×10^6
	Abs	1.000 (0.934)	0.5	0.259	0.136	0.063	0.038	0.078	0.02	0.012	0.006

* PNC = the probable number of cells/ml. Actual counts were performed on the 1 in 128 dilution.

Abs = Absorbance at 600 nm using PPY medium as a blank.

The figures in brackets are the uncorrected absorbance readings obtained when these dilutions were not further diluted.

its cell numbers by a factor of 350 in 8 or 9 generations and the B. melaninogenicus ss. intermedius strain, NCTC 9336, increased its cell numbers by a factor of 600 in 9 generations.

Additions to PY medium

Of the four strains of B. melaninogenicus ss. asaccharolyticus studied in this series of experiments, the two Bangour strains 2296 and 3502 failed to grow after incubation for 5 days in any of the PY based media; no supplement or combination of supplements tested was able to stimulate growth. Bangour strain 3586 however grew reasonably well to 2+ turbidity levels in both PY and PYG media incubated for 5 days. Growth of this strain was not enhanced by addition of ammonium and ferrous sulphate salts but was slightly improved, to 3+ turbidity, by the addition of methionine 7.4 µg/ml. WPH strain 44 grew poorly to ± turbidity levels in PY medium without supplements and to a similar degree in all the supplemented media except for PY containing 2% horse serum where it was inhibited. Growth of WPH 44 was slightly stimulated, to 1+ turbidity, by the addition of disodium succinate and by the addition of tryptophan, ammonia and glutathione together to PY medium. In general however this strain appeared to grow slowly.

Tests on a range of enriched media

Three of the strains tested in the range of supplements to PY medium were chosen for further study in a range of

enriched culture media. The strains, Bangour 2296 and 3502 and WPH 44 had all grown after incubation for 2 days in CMB medium, in CMB enriched with Trypticase (CMB-T), in BM medium, and in PLY medium. All strains had grown slowly in PLY medium to ± or + levels. WPH 44 grew slightly better in CMB and CMB-T media reaching + to ++ levels. It produced ± turbidity in BM medium. The Bangour strains 2296 and 3502 produced 4+ turbidity in CMB, CMB-T and BM media.

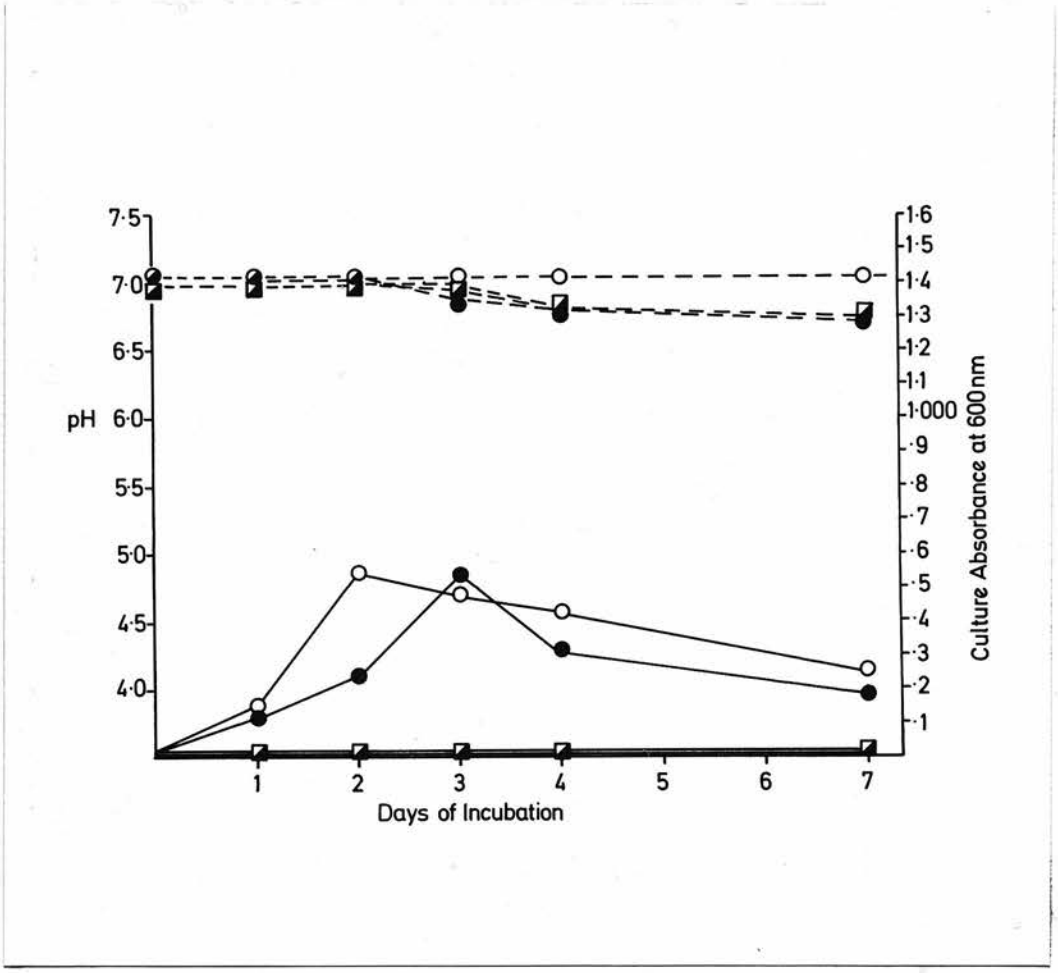
WPH strain 44 grew poorly in PP3 medium but the other test strains failed to grow. None of the strains grew in PPLI or PPLD media.

The influence of balanced salts solution on growth

The possibility that the BSS of Holdeman and Moore (1972) was contributing to the growth failures observed in earlier experiments was examined. Two of the 5 bacteroides strains tested in the ½PPY and ½PPYG media were completely inhibited by the addition of BSS to the media. After incubation for 7 or 8 days both B. melaninogenicus ss. asaccharolyticus strains, NCTC 9337 and Bangour 2296, had failed to grow. However, the Bangour strain also did not grow well in the media without BSS. The NCTC strain produced similar maximum turbidity levels in ½PPY and ½PPYG media reaching spectrophotometric readings of c. 0.500 (see figs. 8a and 8b). Maximum turbidity in ½PPY medium

Figure 8. The influence of a balanced salts solution on the growth of 5 Bacteroides strains, as determined by the changes in culture turbidity and pH, during incubation for a maximum of 7 or 8 days in glucose-free and glucose-enriched $\frac{1}{2}$-strength Proteose peptone-yeast extract medium ($\frac{1}{2}$PPY).

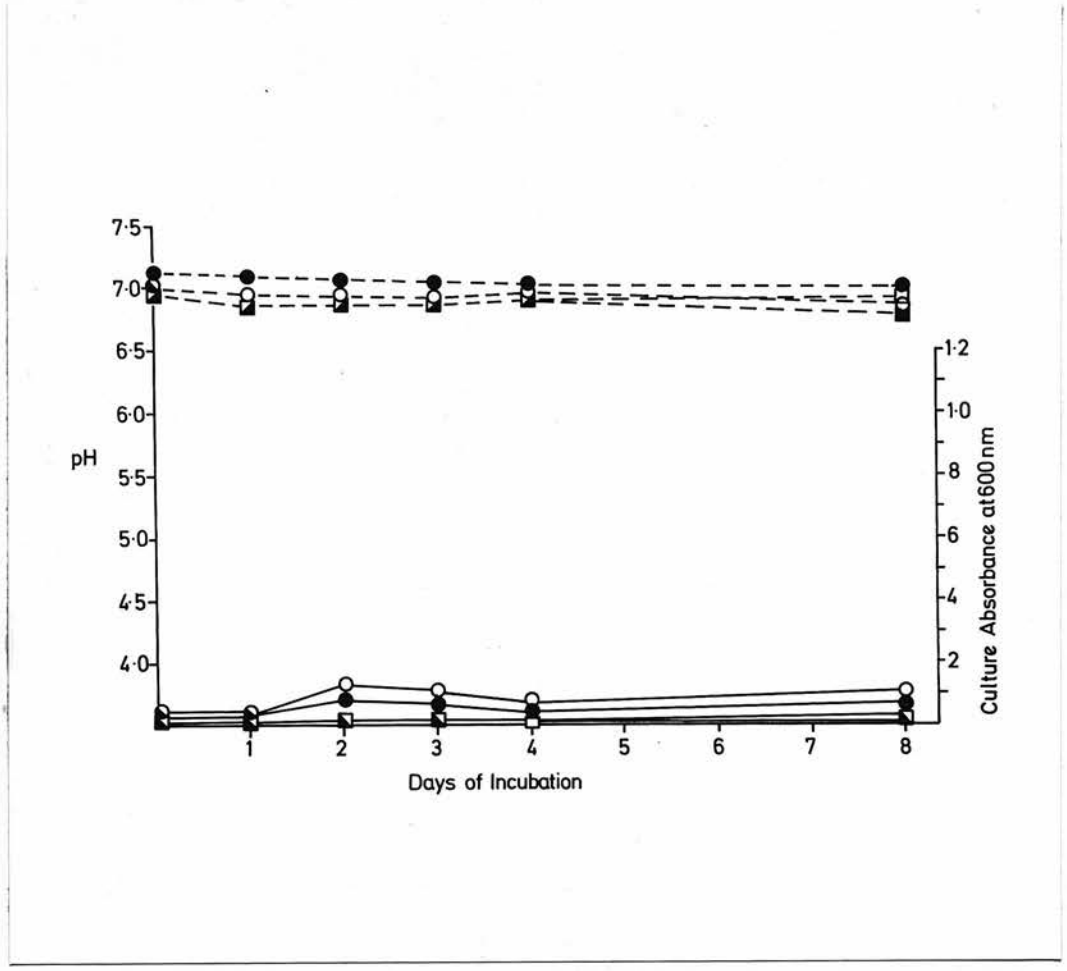
Figure 8a. B. melaninogenicus ss. asaccharolyticus strain NCTC 9337.



KEY

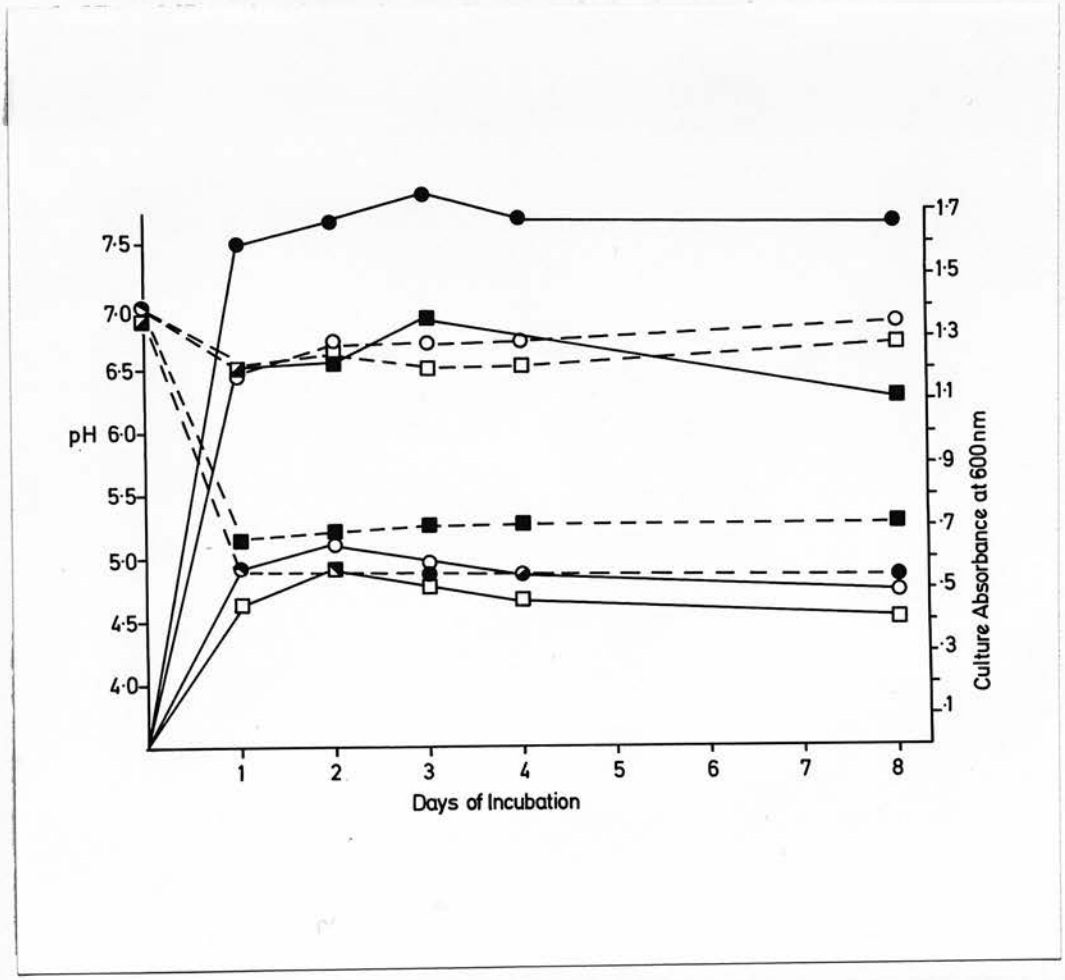
Test:	Culture absorbance at 600 nm	Culture pH	Medium
	○—○	○--○	$\frac{1}{2}$PPY
	●—●	●--●	$\frac{1}{2}$PPYG
	□—□	□--□	$\frac{1}{2}$PPY + BSS
	■—■	■--■	$\frac{1}{2}$PPYG + BSS

Figure 8b. B. melaninogenicus ss. asaccharolyticus strain Bangour 2296.



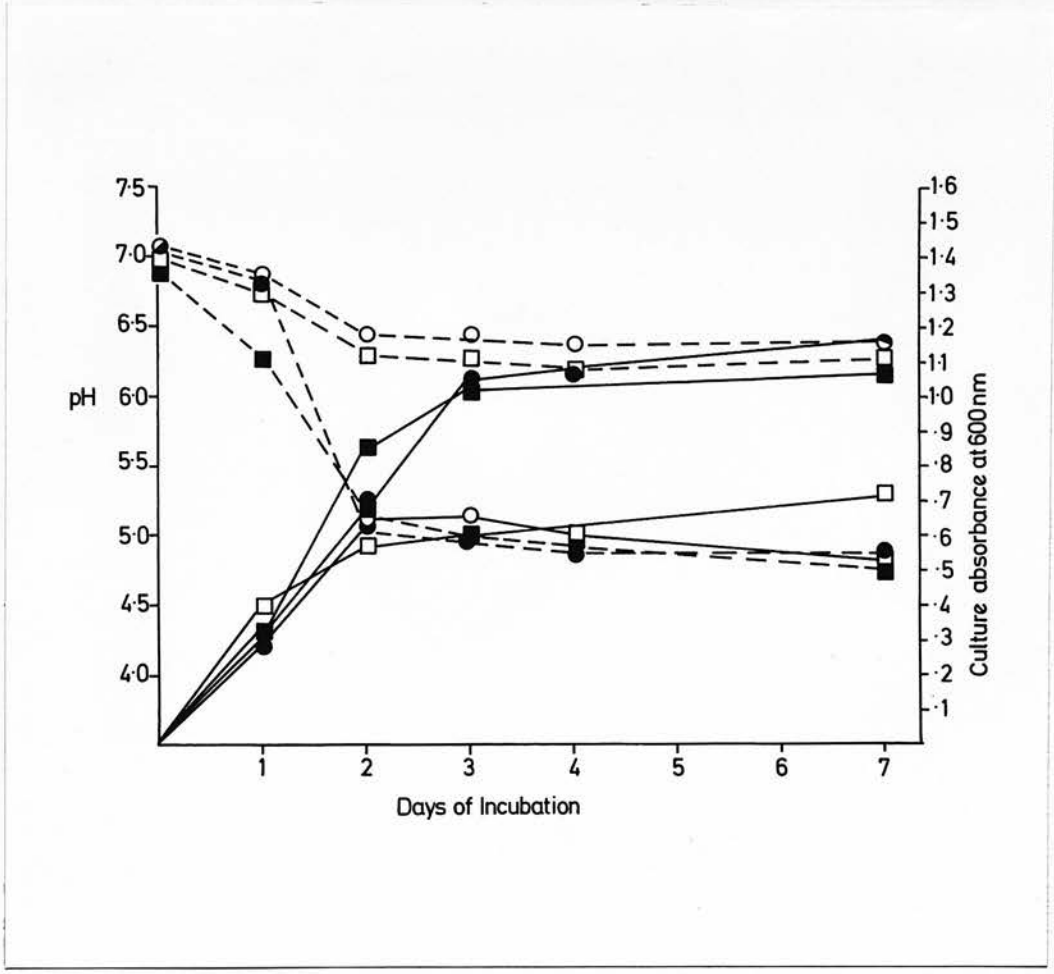
Key. See figure 8a.

Figure 8c. B. fragilis strain NCTC 9344.



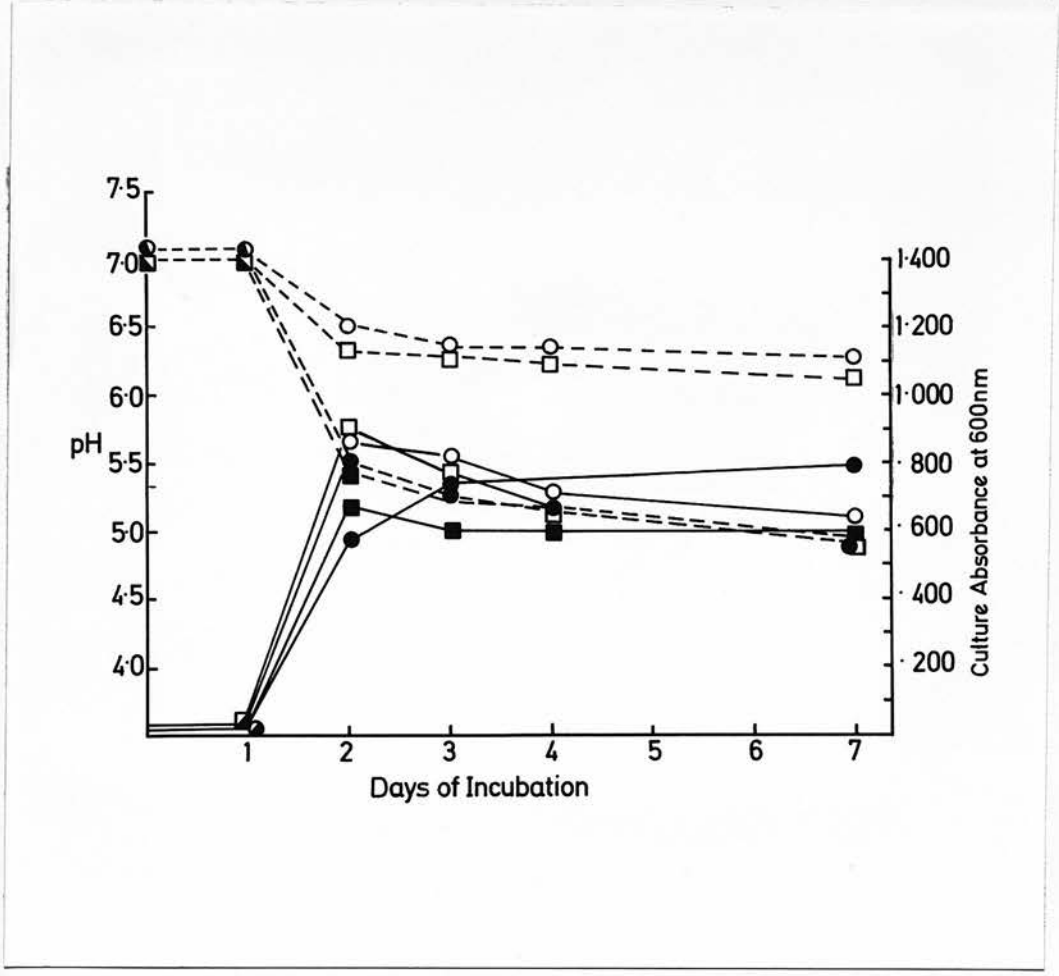
Key. See figure 8a.

Figure 8d. B. melaninogenicus ss. melaninogenicus strain VPI 4196.



Key. See figure 8a.

Figure 8e. B. melaninogenicus ss. intermedius strain NCTC 9336.



Key. See figure 8a.

was achieved after incubation for 2 days but there was a glucose-induced lag in ½PPYG medium and maximum turbidity was reached after 3 days. It was concluded that BSS could have been a significant contributory factor to the earlier growth failures observed but it could not be the sole reason for the failures obtained with PY medium and the proteose peptone-yeast extract media previously tested.

The BSS did not markedly affect the growth of the 3 saccharolytic strains. The major growth differences observed in the test media with these strains were attributable to the presence of glucose in the ½PPYG and ½PPYG-BSS media. All 3 strains grew well in the four test media but produced significantly lower pH values in the glucose-containing media (figs. 8c, 8d and 8e).

The B. fragilis strain NCTC 9344 (fig. 8 c) produced heavy growth in ½PPYG medium reaching a turbidity of 1.7. In the glucose-free media it reached a turbidity of approximately 0.600. The maximum pH fall and maximum turbidity increase occurred in <24 h. The addition of BSS produced minor changes in the pH and turbidity values obtained. The peak turbidity in both BSS-containing media was lower than in the corresponding medium containing NaCl.

The B. melaninogenicus ss. melaninogenicus strain VPI 4196 (fig. 8d) produced a similar pattern of results but

the major pH fall, and corresponding turbidity increase, in $\frac{1}{2}$PPYG medium took 2 to 3 days and the final turbidity levels obtained were lower, reaching values of approximately 1.1. The addition of BSS to the test media did not significantly alter the results obtained.

The B. melaninogenicus ss. intermedius strain NCTC 9336 (fig. 8.e) was also not affected by the addition of BSS to the test media. The pH fall of this strain in the glucose-containing media was slower reaching a final pH of 4.95 after 7 days. Interestingly, the maximum turbidity levels of about 0.900 were reached in 2 days in the glucose-free media.

In general the pH change produced by ultrasonication of culture samples was small ranging from no change to a maximum increase of 0.65 of a pH unit in one sample from a 3 day culture of the B. melaninogenicus ss. asaccharolyticus strain Bangour 2296. Most samples increased the pH by < 0.3 of a unit and slightly larger increases occurred in the cultures where no fermentation had occurred.

In routine fermentation testing of obligate anaerobes a parameter frequently measured to determine whether or not fermentation of a carbohydrate has occurred is the difference in pH developed by the test strain after growth in a glucose-free and glucose-enriched complex medium.

The detailed results in fig. 8 a-e show that the major pH changes resulting from glucose fermentation by the 3 saccharolytic test strains are completed within 4 days in the ½PPYG medium. Table 13 lists the pH values obtained for the 5 test strains in ½PPY and ½PPYG media and summarises the pH differences occurring after 4 days. Two of the strains, B. melaninogenicus ss. intermedius NCTC 9336 and B. melaninogenicus ss. melaninogenicus VPI 4196 produced acid in ½PPY medium and produced a pH fall in that medium of > 0.5 of a unit. In both cases however, the pH difference between ½PPY and ½PPYG media was > 1.0 unit.

Studies with proteose peptone-containing media

The least enriched ½PPY medium supported the growth of the test strains of B. melaninogenicus ss. asaccharolyticus, Bangour 2296 and 3502 and WPH no. 44 to low levels of turbidity, reaching 1+ after incubation for 50 h. Subsequent extensive experience with the medium during vitamin B_{12} growth studies, GLC and fermentation studies confirmed the original observations and showed that the medium would support slow growth of all but the most demanding strains. It was found that increasing the concentration of proteose peptone to 2% improved the growth of a wide range of saccharolytic and asaccharolytic bacteroides strains including the clinically important B. fragilis and the closely related species of

Table 13: The pH changes in a glucose-free and a glucose-enriched complex medium produced by 5 Bacteroides strains incubated for a maximum of 7 or 8 days

Test strain	Days of incubation	pH value* in the test media ½PPY	½PPYG	pH difference after 4 days (± 0.05)
B. melaninogenicus ss. intermedius NCTC 9336	1	7.1	7.1	
	2	6.5	5.5	
	3	6.35	5.25	1.15 (6.3-5.2) acidogenic †
	4	6.3	5.15	
	7	6.3	4.9	
B. melaninogenicus ss. asaccharolyticus NCTC 9337	1	7.1	7.1	
	2	7.0	7.0	
	3	7.1	6.9	0.3 (7.1-6.85)
	4	7.1	6.8	
	7	7.1	6.9	
B. melaninogenicus ss. melaninogenicus VPI 4196	1	6.9	v	
	2	6.45	5.0	
	3	6.45	4.95	1.5 (6.4-4.9) acidogenic
	4	6.35	4.9	
	7	6.4	4.9	
B. fragilis NCTC 9344	1	6.5	4.9	
	2	6.7	4.9	
	3	6.7	4.9	1.8 (6.7-4.9)
	4	6.7	4.9	
	8	6.85	4.9	
B. melaninogenicus ss. asaccharolyticus Bangour 2296	1	7.0	7.1	
	2	6.95	7.1	
	3	6.9	7.05	No change
	4	6.95	7.0	
	7	6.9	7.0	

* pH value ± 0.05 † acidogenic = fall in pH of ½PPY > 0.5 unit

B. thetaiotaomicron, B. distasonis, B. vulgatus and B. ovatus; a large number of B. melaninogenicus strains from the subspecies intermedius, melaninogenicus and asaccharolyticus and the related species of B. oralis, B. ruminicola and the CO_2-dependent B. ochraceus. The addition of glucose further improved the growth rates of many saccharolytic strains, but many of the less demanding strains grew rapidly in the PPY medium without glucose. The addition of 2% serum improved the growth rates of many strains.

The complete BM medium as detailed in table 4 was found to be an excellent culture medium; the test strains of B. melaninogenicus ss. asaccharolyticus, NCTC 9337, Bangour 2296 and 3502 and the WPH strains 41, 45 and 94 all produced > 3+ turbidity within 48 h and reached 4 to 5+ levels after incubation for 96 h. The deletion of trypticase or yeast extract clearly reduced the growth of two test strains but did not affect the growth of the WPH strain no. 44. Increasing concentrations of trypticase or yeast extract in the respective media from 0.25% through 0.5% and 1.0% to 2.0% produced increasingly better growth although the differences between 1% and 2% were minimal. These results are shown in table 14. The substitution of Proteose peptone no. 3 (Difco) for Proteose peptone (Oxoid) in medium II, did not affect the growth rates or recovery of any test strains.

Table 14: The effect of increasing concentrations of Trypticase and yeast extract on the growth of 3 strains of B. melaninogenicus ss. asaccharolyticus

Test strain	Hours of incubation	In medium V* % yeast extract added					In medium VI* % Trypticase added				
		0	0.25	0.5	1.0	2.0	0	0.25	0.5	1.0	2.0
B. melaninogenicus ss. asaccharolyticus Bangour 2296	20	±	±	±	±	±	±	±	< 1+	< 1+	1+
	50	2+	3+	> 3+	4+	5+	1+	2+	< 3+	< 4+	5+
B. melaninogenicus ss. asaccharolyticus Bangour 3502	20	M	±	1+	1+	1+	±	M	M	M	M
	50	±	1+	< 2+	2+	3+	±	±	< 1+	1+	2+
B. melaninogenicus ss. asaccharolyticus WPH 44	20	±	±	±	±	±	±	±	±	±	±
	50	±	±	< 1+	1+	1+	1+	1+	1+	1+	1+

* see table 4 . M = Microscopic growth only.

In general, deletion of horse serum as in medium III, caused an increase in the lag phase of the test strains. The use of 5% horse serum in medium IV did not improve the growth of the test strains in comparison with the results obtained with 2% horse serum in medium II.

Trypticase is an important supplement. The replacement of Trypticase by Tryptone (Difco) in medium VII led to slight reductions in the growth of most test strains after incubation for 48 h. The substitution of 3% casamino acids for Trypticase in serum-free medium VIII or the supplementing of 2% casamino acids with 0.3% Trypticase in serum-free medium X produced less growth than that obtained with serum-free medium III. An increase in the yeast extract supplement did not compensate for the loss of the Trypticase supplement in medium IX; population of all test strains rose to 3 or 4+ levels after incubation for 96 h.

The addition of fresh meat particles to medium III, as in medium XI produced more rapid growth from all test strains (except strain WPH 44) and dense populations of cells were evident within 48 to 96 h. The WPH 44 strain produced results similar to those obtained in all the other media in which it was tested. The strain was found to grow best in Robertson's CMB medium and in that medium containing 1% Trypticase where it reached 1+ levels after 96 h. It grew to the same levels in medium XII, the complete BM medium

enriched with meat particles, but was seemingly unable to grow rapidly to higher population levels in any of the test media studied. All the other test strains of B. melaninogenicus ss. asaccharolyticus grew well in the fully supplemented medium XII to population levels comparable to medium I or II.

Further studies with a range of demanding strains. The test strains of B. melaninogenicus ss. asaccharolyticus Bangour 2296 and 3502 and three of the four demanding WPH strains 34, 36 and 44 were successfully subcultured in the 5 test media indicated in table 15. The WPH strain 37 failed to grow in the serum-free medium III after incubation for 96 h. This strain produced microcolonies and short chains of cells in growing to 3+ levels in the enriched medium IV and grew to 4+ levels after 96 h in the fully supplemented medium XII, Robertson's CMB medium and that medium enriched by 1% Trypticase (CMB-T). The WPH strain 36 developed only $\pm$ levels of growth after incubation for 96 h in media III and IV. It produced 2+ levels in medium XII and 3+ levels in Robertson's CMB and CMB-T media in the same time. The WPH strain 34 grew to 3+ levels after 96 h in media III and IV and 4+ levels in the other test media. The WPH strain 44 did not grow to more than 1+ levels in any of the test media. These results with demanding WPH strains indicate the need for enriched or fully supplemented media.

Table 15: The growth of 6 demanding strains of B. melaninogenicus ss. asaccharolyticus in a range of enriched culture media

Test strains	Hours of incubation	Visual turbidity reached in the test media				
		medium III*	medium IV*	medium XII*	CMB	CMB-T
B. melaninogenicus ss. asaccharolyticus Bangour 2296	20	+	2+	3+	2+	3+
	96	4+	4+	> 4+	4+	5+
B. melaninogenicus ss. asaccharolyticus Bangour 3502	20	±	±	2+	+	2+
	96	< 2+	2+	4+	4+	4+
B. melaninogenicus ss. asaccharolyticus WPH no. 34	20	-	-	±	2+	2+
	96	3+	3+	4+	4+	4+
" no. 36	20	-	-	±	±	±
	96	±	±	2+	3+	> 3+
" no. 37	20	-	-	±	±	±
	96	-	3+	4+	4+	4+
" no. 44	20	±	±	±	±	±
	96	±	±	+	+	+

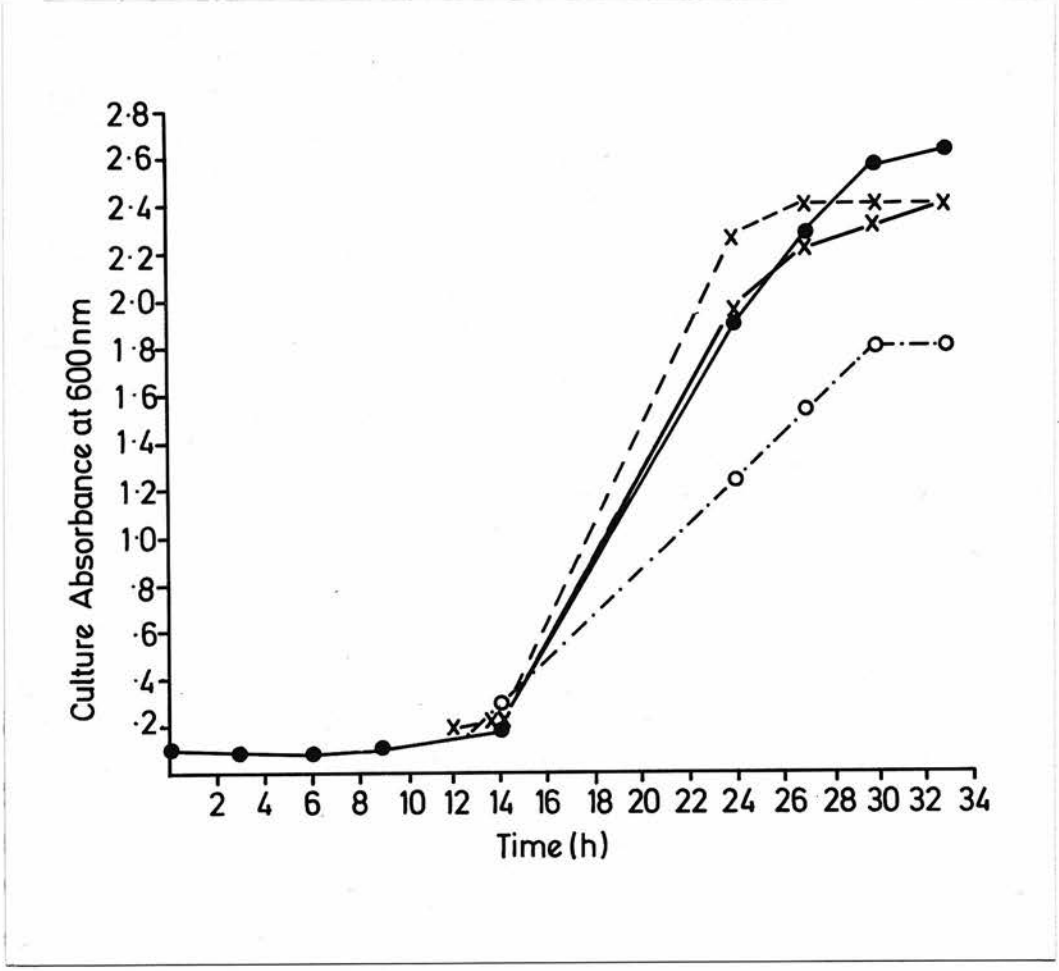
* see table 4

Studies with vitamin B_{12}

During the growth studies it was found that a high concentration of vitamin B_{12} was able to stimulate the growth of the 3 test strains of B. melaninogenicus ss. asaccharolyticus, NCTC 9337 and Bangour 2296 and 3502. When the vitamin was added to ½PPYS medium at a final concentration of 1×10^7 pg/ml the strains achieved high levels of growth similar to that obtained in complete BM medium. Fig. 9 shows the growth response with time of Bangour strain 2296 in BM and ½PPYS media, and in ½PPYS medium containing vitamin B_{12} 1×10^7 pg/ml as determined by culture absorbance at 600 nm in a bulk culture held at an extremely low Eh by gassing with an oxygen-free gas mixture containing 10% CO_2. The test strain was tested in duplicate in BM medium and the rate of growth during the logarithmic phase of growth was identical in both cultures; the vitamin-enriched ½PPYS medium produced a similar profile but the ½PPYS medium without enrichment grew more slowly and did not reach the same final level of turbidity as the other media.

Experiments in anaerobic jars confirmed the findings of the bulk culture experiment. It was found that bacteroides strains belonging to other species or subspecies were not stimulated and the strain designated B. oralis ATCC 15930 (B. melaninogenicus ss. melaninogenicus, Holbrook and Duerden, 1974) was inhibited by the vitamin at 1×10^7 pg/ml in ½PPYS medium. The B. fragilis strains NCTC 9343 and 9344, the

Figure 9. The absorbance of samples taken intermittently from 4 unstirred bulk cultures of the B. melaninogenicus ss. asaccharolyticus strain, Bangour 2296, held at extremely reduced redox potential by continuous gassing with an oxygen-free gas mixture containing 10% CO_2 in 87% N_2 with 3% H_2.



* $\frac{1}{2}PPYS_{12}$ medium contained 1 x 10^7 pg/ml of vitamin B_{12}.

Key.

X——X / ●——● Duplicate cultures in BM medium

X- - -X $\frac{1}{2}PPYS_{12}$ medium

O-·-·O $\frac{1}{2}PPYS$ medium

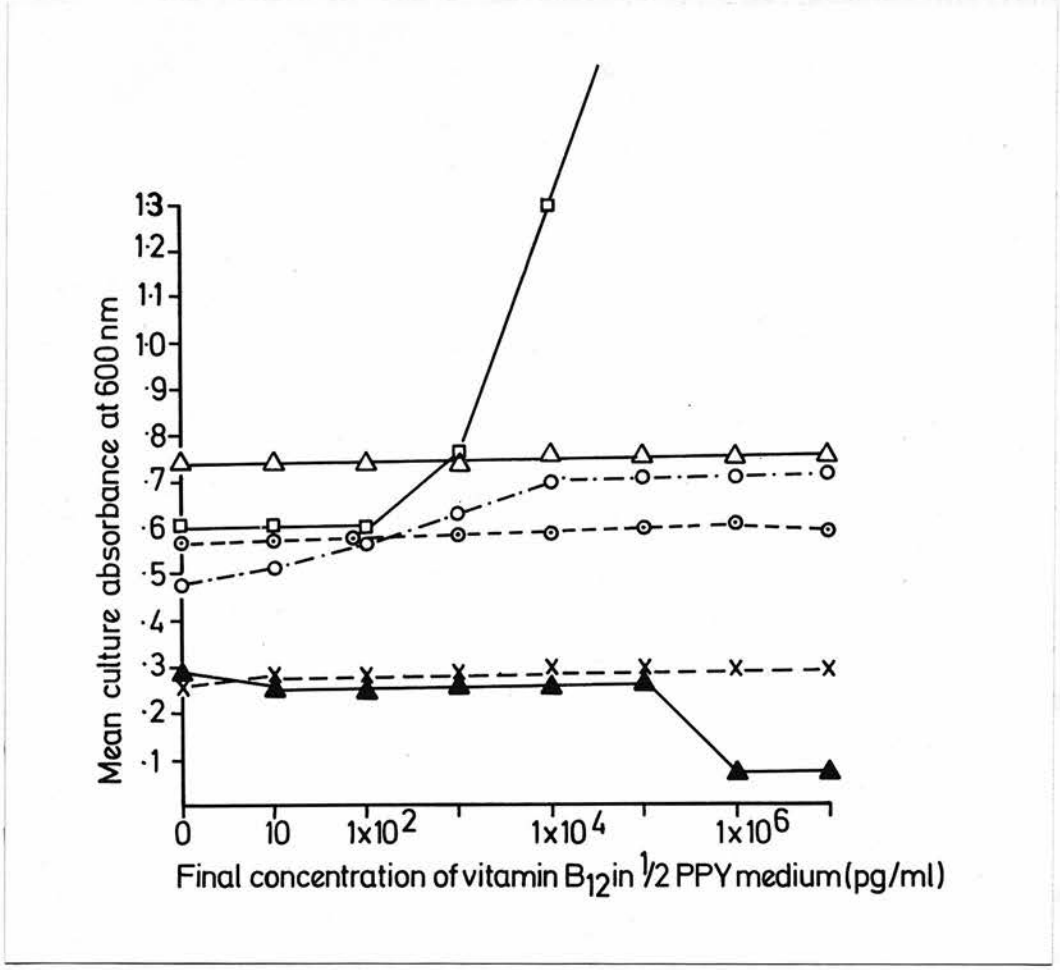
The formulae of the test media are given in Appendix I.

B. thetaiotaomicron strain NCTC 10582 and the B. vulgatus strain NCTC 10583 had all grown slightly better in the vitamin-enriched medium, but the differences were marginal after incubation for 24 h.

Horse serum was found to produce an additive effect on the growth stimulation caused by vitamin B_{12}. On one occasion the type strain of B. melaninogenicus ss. asaccharolyticus NCTC 9337 produced an absorbance reading of 0.365 in $\frac{1}{2}$PPY medium; in $\frac{1}{2}PPY_{12}$ medium it was 0.63 and in $\frac{1}{2}PPYS_{12}$ it was 0.96 after incubation for 23 h. As a result of these observations, serum was not added to the media used in subsequent studies involving the vitamin.

The inhibition of the B. oralis strain ATCC 15930 by the vitamin at 1×10^7 pg/ml led to an examination of the effect of different concentrations of the vitamin on the growth of 6 B. melaninogenicus strains in $\frac{1}{2}$PPY medium. The detailed results are shown in Fig. 10. After incubation for 90 h the B. oralis strain ATCC 15930 was inhibited by concentrations of 1×10^5 pg/ml or more. No other test strain was inhibited by addition of the vitamin. The B. melaninogenicus ss. asaccharolyticus strain Bangour 2296 was only slightly stimulated in this experiment after incubation for 20 h, achieving best results in the test media containing 1×10^4 pg/ml, or more, of the vitamin. The other asaccharolytic strain, Bangour 3502, produced best results in these concentrations after incubation for

Figure 10. The mean culture absorbance of 6 strains* of B. melaninogenicus incubated for varying intervals in $\frac{1}{2}$PPY medium and that medium enriched with 7 different concentrations of vitamin B_{12}.



* The test strains were:

B. melaninogenicus ss. intermedius NCTC 9336 and

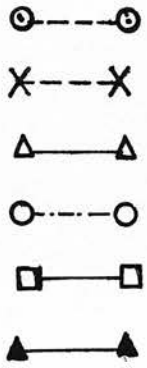
" " " " " 9338

B. melaninogenicus ss. melaninogenicus VPI 4196

B. melaninogenicus ss. asaccharolyticus Bangour 2296 and

" " " " " 3502

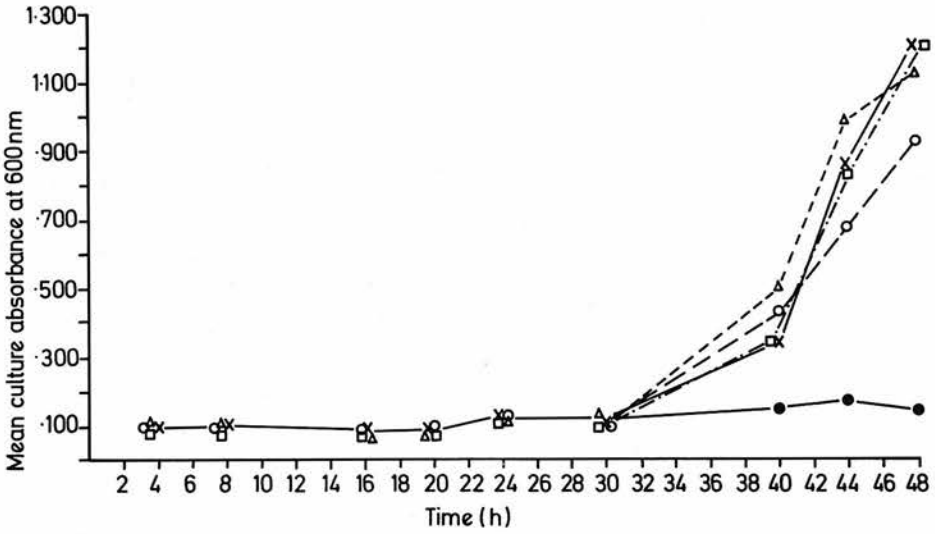
B. oralis (B. melaninogenicus ss. melaninogenicus) ATCC 15930



44 h. The growth of the 3 remaining, saccharolytic, strains of B. melaninogenicus was not stimulated by addition of the vitamin.

The growth of the B. melaninogenicus ss. asaccharolyticus strain Bangour 2296 was also tested in PPY medium and in the same medium containing vitamin B_{12} at concentrations ranging from 1×10^4 to 1×10^7 pg/ml, with incubation for intervals of time up to a maximum of 48 h (Fig. 11). The growth rates with each of the vitamin B_{12}-containing cultures were similar and confirmed the earlier observation that growth of this strain was best at concentrations of vitamin B_{12} of 1×10^4 pg/ml or more. Microscopy on selected cultures showed that the morphology of the cells was not significantly altered by addition of the vitamin to PPY medium and total cell counts performed on the cultures in PPY medium and on the cultures containing 1×10^5 pg/ml of the vitamin showed that the turbidity increases resulted from increased cell numbers. The total cell counts in the two media were virtually identical after incubation for 40 h. After incubation for 48 h however, there were significant differences. The total cell count in vitamin-free PPY medium was 1.4×10^8 cells/ml and in the vitamin enriched medium it was 2.28×10^9 cells/ml. This represents more than a 16 fold difference in the cell numbers and shows clearly the stimulatory effect of the vitamin on this strain.

Figure 11. The mean culture absorbance of B. melaninogenicus ss. asaccharolyticus strain Bangour 2296 in PPY* medium and in that medium enriched with 4 different concentrations of vitamin B_{12} incubated for intervals of time up to a maximum of 48 h.



* see Appendix I

Final concentrations of vitamin B_{12} in the test media:

- PPY medium without added vitamin
- PPY + 1 x 10^4 pg/ml of B_{12}
- PPY + 1 x 10^5 pg/ml of B_{12}
- PPY + 1 x 10^6 pg/ml of B_{12}
- PPY + 1 x 10^7 pg/ml of B_{12}

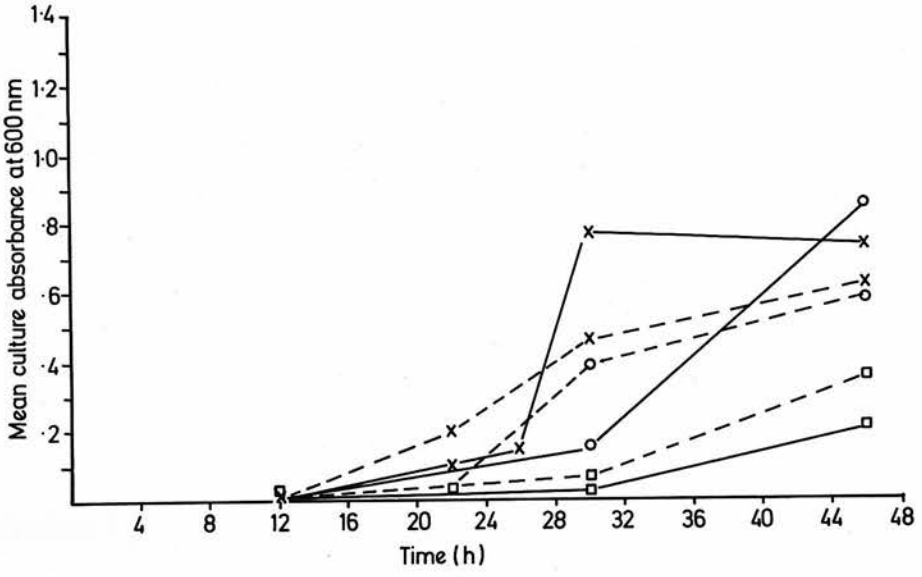
The 3 strains of B. melaninogenicus ss. asaccharolyticus were also tested in ½PPY, PPY and BM-S media and in the same media enriched with vitamin B_{12} at 1 x 10^5 pg/ml. Growth curves were prepared by plotting the mean absorbance of separate duplicate cultures incubated for varying periods of time up to a maximum of 46 h. Figs. 12a, b and c show the plot of mean culture absorbance against time for each test strain in the 6 media tested. With only one exception, growth rates were higher in the vitamin-enriched media. The type strain NCTC 9337 produced slightly better growth after 46 h in the vitamin-free cultures in ½PPY medium but the slope of the growth curves obtained with the ½PPY and the ½PPY_{12} media were virtually identical indicating that no significant stimulation had occurred. The results in Fig. 12a show that the strain is clearly stimulated in the vitamin B_{12}-enriched PPY and BM-S media.

The amino acid DL methionine was unable to stimulate the growth of the 3 test strains of B. melaninogenicus ss. asaccharolyticus when added to ½PPY or PPY media at concentrations of 7.4, 74 or 740 µg/ml. The strains grew well in the media containing the amino acid although growth in the ½PPY media was generally at low levels after incubation for 42 h.

Evidence of the rapid uptake of vitamin B_{12} from PPY medium by the test strains of B. melaninogenicus ss.

Figure 12. The mean culture absorbance of 3 strains of B. melaninogenicus ss. asaccharolyticus in separate duplicate cultures incubated for intervals of time up to a maximum of 46 h to show the effect of addition of vitamin B_{12}, 1.0 x 10^5 pg/ml, to a range of complex media.

Figure 12a. B. melaninogenicus ss. asaccharolyticus, NCTC 9337.

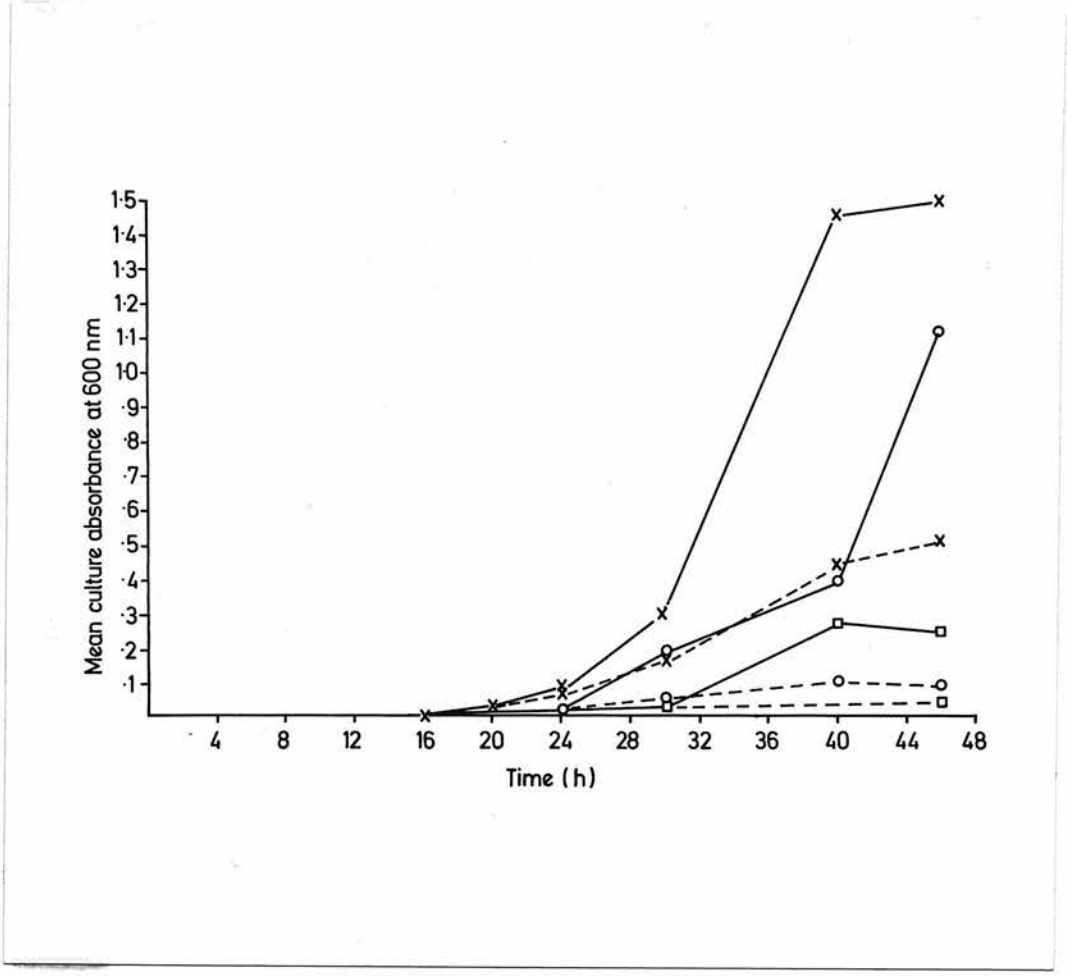


Key.

- ½PPY + 1 x 10^5 pg/ml of B_{12}
- ½PPY without added vitamin
- PPY + 1 x 10^5 pg/ml of B_{12}
- PPY without added vitamin
- BM-S + 1 x 10^5 pg/ml of B_{12}
- BM-S without added vitamin

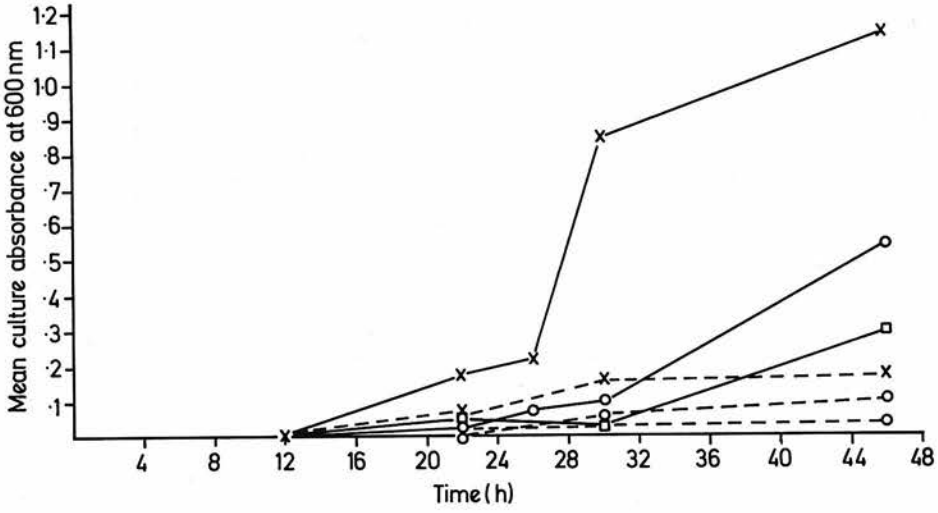
The formulae of the media is given in Appendix I.

Figure 12b. B. melaninogenicus ss. asaccharolyticus, strain Bangour 2296.



for key to media see figure 12a.

Figure 12c. B. melaninogenicus ss. asaccharolyticus, strain Bangour 3502.



for key to media see figure 12a.

asaccharolyticus, NCTC 9337 and Bangour 2296 and 3502, was obtained in two separate radioactive labelling experiments using ^{57}Cobalt-labelled vitamin B_{12} (cyanocobalamin). The results (table 16) show that all the test strains behaved similarly under the test conditions maintaining good correlation in their detailed results. The duplicate cultures of Bangour strain 3502 examined after incubation for 24 h showed a discrepancy which was due to a growth failure in one of the tubes. Little or no label was lost from the culture medium in the first 4 h of anaerobic incubation, but > 70% was lost within a 24-h period. The losses in the following 24-h period were small by comparison.

Good growth was obtained in all the vitamin B_{12}-enriched cultures, except for the single tube of strain Bangour 3502 incubated for 24 h. Examination of the 3 vitamin-free cultures incubated for 48 h in parallel with vitamin-enriched cultures showed that the vitamin present in the test cultures at 1×10^{3} pg/ml did not stimulate the growth of any strain.

The results of the percentage recovery experiment, detailed in table 17, confirmed the findings of the previous experiment and showed that the loss of the vitamin from the medium was caused by cellular uptake. The total recovery figures were consistently high and the amount of label lost in the saline washes was uniformly low. In this experiment the test strains took up an average of 75.9%

Table 16: The loss of ^{57}Cobalt-labelled vitamin B_{12} from the culture supernates of duplicate cultures in PPY medium of 3 strains of B. melaninogenicus ss. asaccharolyticus

Test strain	Incubation time (h)	Sample	Counts per second (cps)	Amount of vitamin remaining in the supernate (ng)	Loss of label from the supernate (%)
		Uninoculated medium	755	10.000*	0
B. melaninogenicus ss. asaccharolyticus NCTC 9337	4	1	690	9.139	8.6
		2	714	9.457	5.4
	24	1	86	1.139	88.6
		2	119	1.576	84.2
	48	1	90	1.192	88.1
		2	97	1.285	87.1
B. melaninogenicus ss. asaccharolyticus Bangour 2296	4	1	769	10.185	0
		2	833	11.033	0
	24	1	222	2.940	70.6
		2	N.T.	N.T.	N.T.
	48	1	102	1.351	86.5
		2	104	1.378	86.2
B. melaninogenicus ss. asaccharolyticus Bangour 3502	4	1	769	10.185	0
		2	769	10.185	0
	24	1	526	6.967	30
		2	114	1.510	84.9
	48	1	189	2.503	74.9
		2	N.T.	N.T.	N.T.

* The uninoculated medium was prepared to contain 10 ng of labelled vitamin B_{12}, cyanocobalamin, in each 10 ml.

N.T. = not tested.

Table 17: The percentage recovery of ^{57}Cobalt-labelled vitamin B_{12} from triplicate cultures in PPY medium* of 3 strains of B. melaninogenicus ss. asaccharolyticus

Test strain	Sample	Amount of ^{57}Co-B_{12} in supernate (cpm)	Recovery in supernate (%)	Amount of ^{57}Co-B_{12} in saline wash (cpm)	Recovery in wash (%)	Amount of ^{57}Co-B_{12} in cells (cpm)	Recovery in cells (%)	Total recovery (%)
B. melaninogenicus ss. asaccharolyticus Bangour 2296	i	39.60	15.68	2.87	1.14	187.46	74.24	91.06
	ii	43.96	17.41	2.66	1.05	197.94	78.40	96.86
	iii	45.98	18.21	2.87	1.14	188.04	74.47	93.82
B. melaninogenicus ss. asaccharolyticus NCTC 9337	i	47.06	18.64	3.86	1.53	192.31	76.17	96.34
	ii	47.62	18.86	4.71	1.87	185.54	73.48	94.21
	iii	46.51	18.42	4.13	1.64	187.14	74.12	94.18
B. melaninogenicus ss. asaccharolyticus Bangour 3502	i	20.41	8.08	6.35	2.51	202.41	80.17	90.76
	ii	37.04	14.67	3.70	1.47	202.66	80.26	96.40
	iii	43.96	17.41	7.45	2.95	183.28	72.59	92.95
Uninoculated Medium		252.49						100.00

* All cultures incubated anaerobically for 48 h.

of the label after incubation for 48 h. As each culture contained vitamin B_{12} 1 x 10^3 pg/ml, this figure represents an average utilisation of 759 pg/ml, most of which apparently occurred in the first 24 h of growth.

There was some variation in the response of the 3 asaccharolytic test strains to the inclusion of vitamin B_{12} in the $\frac{1}{2}$PPY or PPY media. In occasional experiments one or other of the test strains failed to show good growth stimulation when compared with the vitamin-free control media. Occasionally there were discrepancies between duplicate cultures with one culture stimulated and one showing poor stimulation or none at all. The causes of this variability were not determined but it did not seem to be due to any variation in the age or condition of the starting inoculum.

Similar variation was also seen in the minimum period of incubation required to obtain good growth in the test media. In some experiments the test strains grew well in BM and PPY media, with or without added horse serum, after incubation for 24 h, but more frequently 40 to 48-h incubation was required. There were fairly consistent differences in the growth rates of the asaccharolytic test strains throughout the growth studies despite changes in the test media. The NCTC strain 9337 grew well in all the test media with little difficulty; the Bangour strain 2296 grew

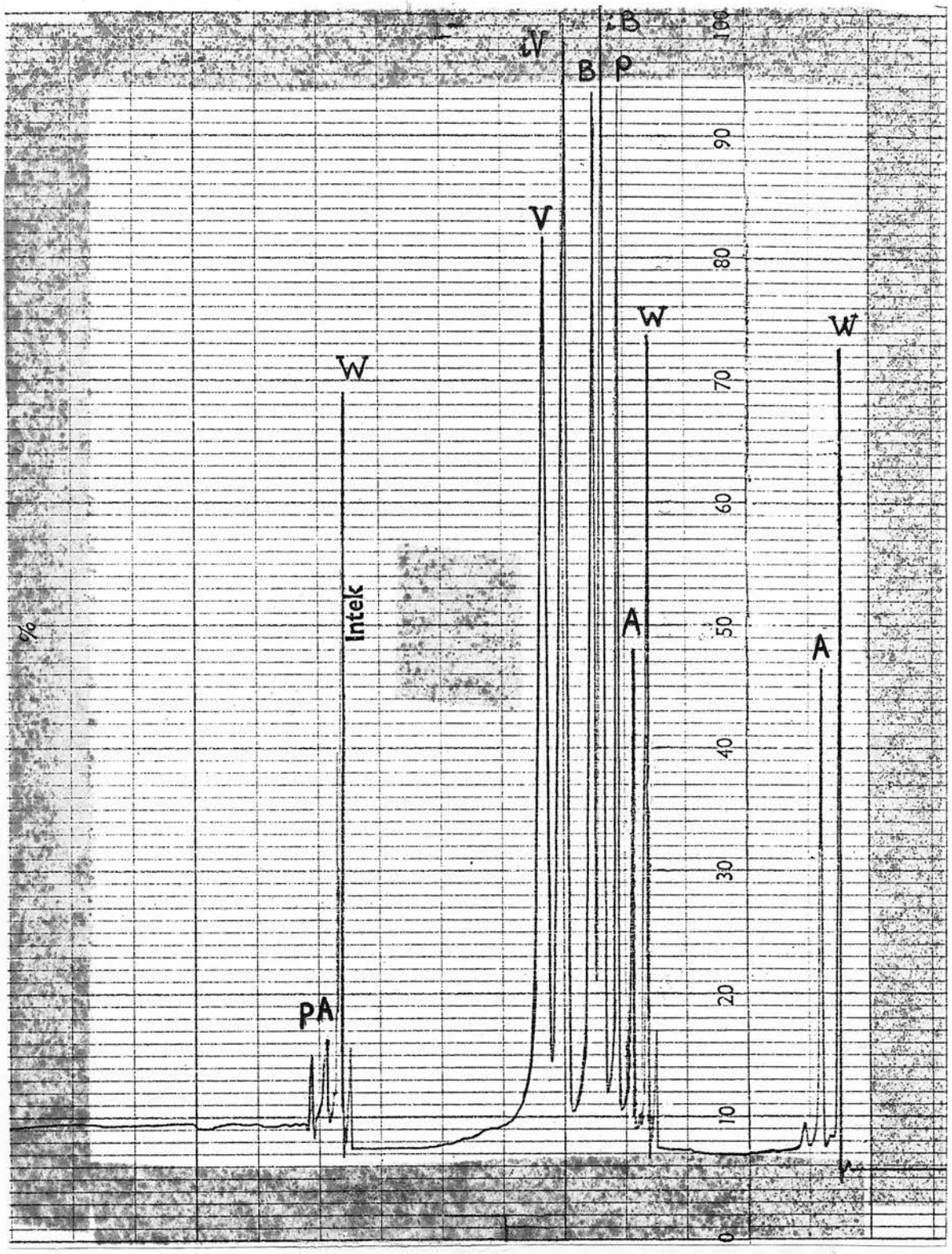
slowly in ½PPY medium, and moderately well in PPY medium; in the enriched BM medium or the vitamin B_{12}-enriched cultures, it grew rapidly to high turbidity levels. The Bangour strain 3502 was more demanding and grew slowly in ½PPY medium without reaching high turbidity levels. In the richer media it showed a similar pattern of results to that of the other Bangour strain.

Gas-liquid chromatography in the identification of the Bacteroidaceae

Evaluation of the column packing and the analytical methods

Preliminary trials with a column packing consisting of 20% polyethylene glycol 20M on 100-120 mesh Diatomite C-AW treated with phosphoric acid (Pye Unicam Ltd) had shown that the use of this packing was limited by its relatively low maximum operating temperature and consequently excessively long ($\geq$ 30 min) analysis times for the short chain, C_2-C_5 volatile fatty acids. By contrast, the Chromosorb 101 packing finally adopted for use enabled fast, efficient separation of these acids. At a carrier gas flow rate of 35 ml/min the retention time of n-valeric acid was typically 9 min. Fig. 13 illustrates a typical volatile fatty acid profile obtained from the standard acid mixture used routinely and shows typical profiles of the fully supplemented BM and PPYSG media. Other media used

Figure 13. Typical volatile fatty acid profiles obtained from the standard mixture of pure acids and from uninoculated PPYSG and BM-S media.



Sample injected: PPYSG medium VFA BM-S medium

Key: W = water, A = acetic acid, P = propionic acid, iB = iso-butyric acid, B = n-butyric acid, iV = iso-valeric acid, V = valeric acid, VFA = volatile fatty acid standards.

in the studies produced profiles similar to that for the PPYSG medium.

Column stability was good with the operating conditions established. The separation of the nC_4-iC_4 peaks was adequate but complete separation at the baseline was not achieved. Similarly the separation of the nC_5-iC_5 peaks was incomplete. Peak symmetry of the C_2-C_5 acids was consistently good and minimal tailing occurred during prolonged use of the column. Lactic and succinic acids were also detected in the volatile analyses at concentrations of 5 µmols/ml or above. The lactic acid peak first appeared after approximately 12.5 min. and was a low spread out peak with substantial tailing. Succinic acid was usually detected after 30 to 35 minutes retention depending on minor variations in the flow rate of the carrier gas. This peak was also asymmetrical.

The reproducibility of peak heights with a single acid standard, in both volatile and non-volatile analyses, was excellent but the sensitivity of response to acetic acid tended to fall off with increasing age of the column packing. This was attributed to the contamination occurring at the injection site on the column resulting from the injection of untreated culture supernates during volatile analyses. Periodic replacement of the top 3 to 4 cm of packing usually allowed normal operation to continue.

Eventually after 2 or 3 such replacements the column packing was completely replaced with fresh material.

Repeated trials with cationic and anionic exchange resins, recommended by Carlsson (1973) for cleaning up culture samples prior to injection, showed that the use of these materials would prevent column contamination but only at the expense of a loss in effective peak height at the 1×10^2 attenuation, as a result of the added dilution. Elution problems also led to poor reproducibility in quantitative estimations with acetic and propionic acids particularly affected.

Comparison of media for GLC analyses

The growth studies with strains of B. melaninogenicus ss. asaccharolyticus showed that the PYG medium (Holdeman and Moore, 1972) was unsatisfactory for the culture of some strains of bacteroides, whereas good growth was obtained in media based on Proteose peptone /BM, $\frac{1}{2}$PPY and PPY media supplemented on occasion with horse serum, glucose or vitamin B_{12}; see Materials and Methods/. In further trials a wide range of Bacteroides spp. were successfully subcultured in BM, $\frac{1}{2}$PPY, $\frac{1}{2}$PPY$_{12}$, PPY, PPYG and PPYSG media (see Appendix I). There were no growth failures, and freshly isolated strains grew well from small inocula although some asaccharolytic strains required 3-4 days to do so. Some cultures of asaccharolytic strains produced

relatively little turbidity, but good growth was confirmed by microscopy.

Initial studies with wild strains. BM medium was used for the GLC analyses on 30 of 72 subgingival isolates, and the remaining 42 strains were tested in $\frac{1}{2}PPY_{12}$ medium; 32 of the 72 strains were independently identified as B. melaninogenicus ss. melaninogenicus, and 40 strains were ss. intermedius. There were no differences in the ranges or relative amounts of fatty acids produced by the two subspecies in the two media. All 72 strains produced succinic and acetic acids in significant quantities (> 10 µmols/ml) and all produced minor amounts of iso-valeric acid with trace amounts of iso-butyric acid. Most strains produced traces of propionic acid but none produced n-butyric acid. The two subspecies were generally distinguished by the amount of lactic acid produced: 38 of 40 ss. intermedius strains produced 60-150 (median 95) µmols/ml, whereas 28 of 32 ss. melaninogenicus strains produced 4-60 (median 38) µmols/ml in single analyses on 3-day cultures. The large amount of acetic acid in sterile BM medium (fig. 13) led to its exclusion from further quantitative GLC studies.

Studies with reference strains grown in a range of derived media. The changes in fatty acid production by five reference strains of the B. melaninogenicus-B. oralis

group in the different media are shown in table 18 . No valid type strain of B. oralis is currently available, but strain NP 333 shares many of the characteristics originally ascribed to this species (Loesche, Socransky and Gibbons, 1964). The three pigmented saccharolytic strains produced characteristic fatty acid profiles that were consistent in all the media; succinic and acetic acids were the major products. n-Butyric acid was not produced by any saccharolytic strains, but it was a significant product of the asaccharolytic strain NCTC 9337. This strain also differed from the four saccharolytic strains by not producing succinic acid in any medium.

The incorporation of glucose in the culture medium induced only minor changes in the range of acids produced. Strain NP 333 produced iso-valeric and lactic acids only in glucose free media. Glucose stimulated acetic acid production by the strains labelled VPI 4196, ATCC 15930 and NP 333. The NCTC strain 9336 produced less acetic, lactic, iso-butyric and iso-valeric acids in glucose enriched media; this was not attributable to less growth. Glucose did not affect acid production by the NCTC strain 9337.

The addition of serum had little or no effect on growth or fatty acid production by the four saccharolytic strains. The effect of serum on the NCTC strain 9337 was inconsistent:

Table 18: Production of volatile fatty acids by five reference strains of Bacteroides spp. in six different Proteose peptone media*

Test strain	Test medium*	Approx. concentration of the stated fatty acid produced †						
		A	P	iB	B	iV	L	S
B. melaninogenicus ss. intermedius NCTC 9336	PPY	21	<1	2	0	3	110	26
	PPYS	25	<1	2	0	5	171	46
	½PPY12	15	<1	1	0	3	90	25
	½PPY12S	15	<1	2	0	3	131	26
	PPYG	16	<1	<1	0	1	62	35
	PPYSG	22	<1	<1	0	2	64	37
B. melaninogenicus ss. melaninogenicus VPI 4196	PPY	11	<1	1	0	2	13	35
	PPYS	11	<1	1	0	2	14	37
	½PPY12	6	<1	1	0	1	14	22
	½PPY12S	6	<1	<1	0	1	13	25
	PPYG	20	<1	1	0	1	8	4
	PPYSG	20	1	1	0	1	6	39
B. melaninogenicus ss. melaninogenicus ATCC 15930	PPY	10	<1	0	0	0	0	21
	PPYS	9	1	0	0	0	0	17
	½PPY12	5	0	0	0	0	0	14
	½PPY12S	10	<1	0	0	0	11	11
	PPYG	18	1	0	0	0	0	26
	PPYSG	14	0	0	0	0	0	27
B. oralis NP 333	PPY	6	<1	0	0	1	32	17
	PPYS	4	<1	0	0	<1	11	17
	½PPY12	4	<1	0	0	<1	9	8
	½PPY12S	3	<1	0	0	<1	9	10
	PPYG	6	1	0	0	0	0	18
	PPYSG	10	1	0	0	0	0	22
B. melaninogenicus ss. asaccharolyticus NCTC 9337	PPY	21	5	3	12	6	144	0
	PPYS	25	4	2	11	5	121	0
	½PPY12	12	9	1	2	2	64	0
	½PPY12S	19	12	2	4	3	145	0
	PPYG	25	6	3	13	6	148	0
	PPYSG	17	1	1	6	3	148	0

* see Methods section for details of these media.

† Fatty acid concentration expressed in μmol per ml estimated by measurement of peak height with reference to aqueous standards corrected for values obtained with medium only. Results are those of single analyses for each sample.

A = acetic; P = propionic; iB = iso-butyric; B = n-butyric; iV = iso-valeric; L = lactic; S = succinic.

production of acetic acid was slightly enhanced by serum in the absence of glucose; growth and production of propionic acid were enhanced when serum and vitamin B_{12} were added; production of other acids was not affected.

The effect of vitamin B_{12} supplement. The NCTC strain 9337 was grown for 23 h in $\frac{1}{2}$PPY, $\frac{1}{2}$PPY$_{12}$, $\frac{1}{2}$PPY$_{12}$S, PPY and PPYS medium. Fig. 14 shows the volatile fatty acid profiles obtained from the combined acid standard and the cultures, and table 19 shows the concentrations of the individual volatile fatty acids achieved in the various media. The production of acetic, iso-butyric and iso-valeric acids was directly proportional to turbidity. The production of propionic acid was significantly higher in the $\frac{1}{2}$PPY$_{12}$ and $\frac{1}{2}$PPY$_{12}$S media. Serum and vitamin B_{12} independently stimulated growth; together they produced an additive effect. The production of n-butyric acid was slightly inhibited by vitamin B_{12}.

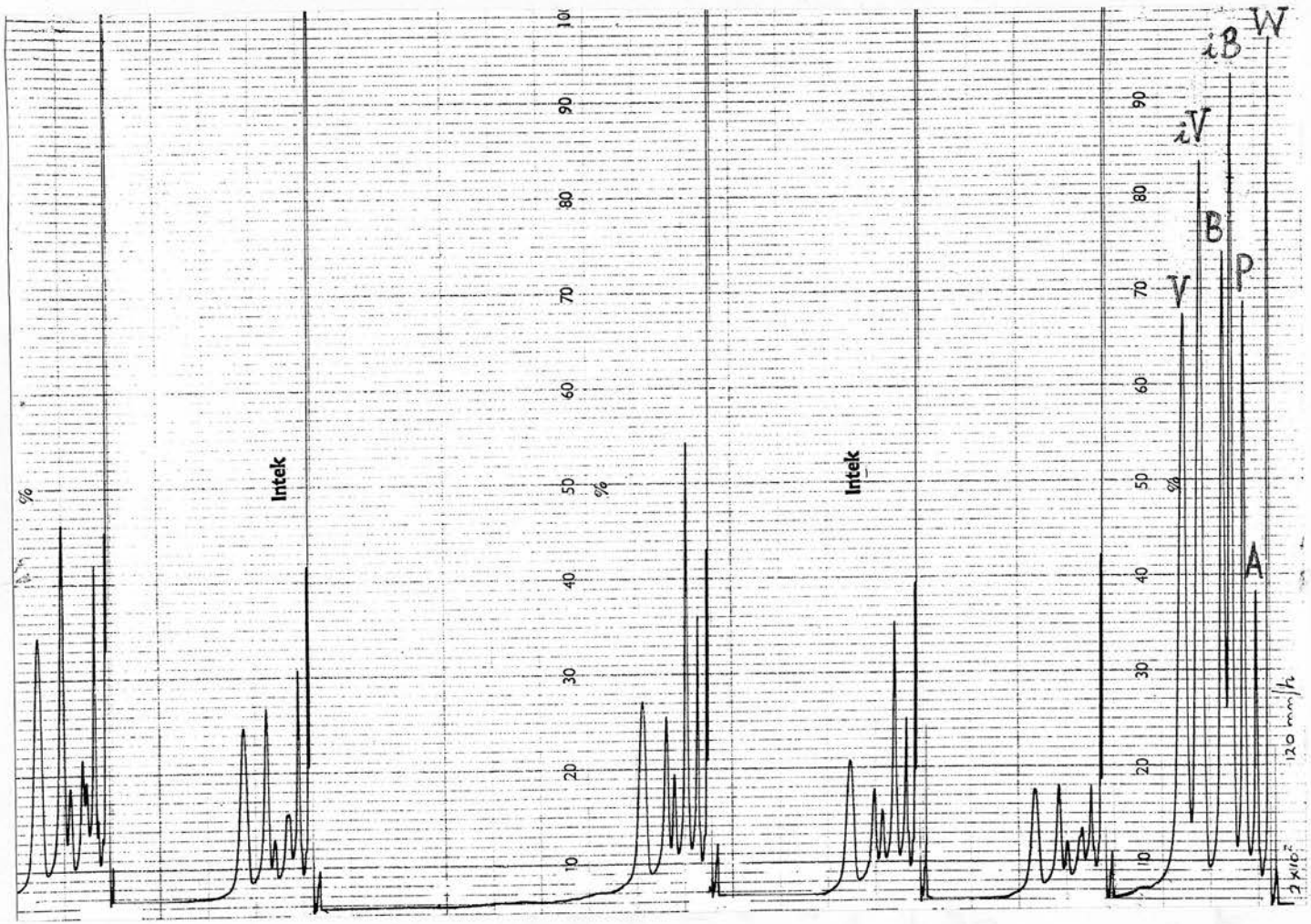
Definitive studies of fatty acid production

Fatty acid profiles of reference strains grown in PPYSG. Table 20 shows the fatty acids produced by 12 reference strains of Bacteroidaceae in single analyses on 2-day cultures in PPYSG medium. The Bacteroides strains all produced similar fatty acid profiles that were clearly distinct from the profiles of Fusobacterium and Leptotrichia strains. All Bacteroides strains produced succinic,

Legend to figure 14

FIGURE - The volatile fatty acid profiles of B. melaninogenicus ss. asaccharolyticus strain NCTC 9337, grown for 23 h in 5 different media, compared with the plot of a combined acid standard (VFA) to show the effect of vitamin B_{12} (cyanocobalamin) and horse serum on growth and acid production. The various media are indicated at the bottom of the chart and the Turbidity (T) of each test culture is given at the top of the chart. The combined acid standard contained 10 µmols/ml of each of the following acids: Acetic (A), Propionic (P), iso-Butyric (iB), n-Butyric (nB), iso-Valeric (iV) and n-Valeric (nV). Details of the media used and the chromatographic conditions are given in the Methods section. Chart speed = 120 mm per h.

Absorbance of test cultures at 600nm:	1.15	0.79		0.96	0.63	0.365



KEY:

V = n-valeric
iV = iso-valeric
B = n-butyric
iB = iso-butyric
P = Propionic
A = Acetic acid
W = Water
VFA = Volatile fatty acid standards

Test Medium	PPYS	PPY	½PPY12S	½PPY12	½PPY	VFA

FIGURE 14

Table 19: The volatile fatty acids produced by strain NCTC 9337 in 23 h cultures of Proteose peptone media

Medium	Turbidity at 600 nm	Approximate concentration (μmols per ml) of the stated acid*				
		A	P	iB	B	iV
½PPY	0.365	0.87	<0.2	0.44	1.52	1.44
½PPY12	0.63	4.2	3.04	0.68	1.44	1.8
PPY	0.79	6.1	<0.2	0.56	2.88	2.24
½PPY12S	0.96	7.3	6.0	1.06	2.48	2.6
PPYS	1.15	9.6	0.7	1.06	5.76	3.37

* see Footnote table 18.

Table 20: Approximate concentration of fatty acids produced by 12 reference strains of the genera Bacteroides - Fusobacterium - Leptotrichia in 2-day cultures in PPYSG medium

Strain identity	Strain No.	Approx. concentration (µmols per ml) of the stated acid*						
		A	P	iB	B	iV	L	S
Bacteroides fragilis	NCTC 9344	22	1	0	0	< 1	4	35
" "	NCTC 8560	21	1	0	0	< 1	4	33
" thetaiotaomicron	NCTC 10582	21	< 1	0	0	0	3	26
" "	ATCC 8492	12	< 1	0	0	0	2	19
" ovatus	ATCC 8483	21	1	0	0	< 1	3	33
" distasonis	ATCC 8503	4	4	< 1	0	< 1	7	22
" vulgatus	ATCC 8482	5	5	< 1	0	< 1	17	18
" "	NCTC 10583	20	1	0	0	0	0	35
Fusobacterium necrogenes	NCTC 10723	6	2	0	13	0	2	0
" polymorphum	NCTC 10562	18	2	0	14	0	3	2
" necrophorum	NCTC 10575	7	5	0	12	0	3	0
Leptotrichia buccalis	NCTC 10249	1	0	0	0	0	22	2

* see footnote table 18.

acetic, lactic and propionic acids. The Fusobacterium strains produced moderate amounts of n-butyric acid and small quantities of lactic and propionic acids but not iso-butyric or iso-valeric acids; one strain produced a trace of succinic acid. The Leptotrichia isolate produced traces of acetic and succinic acids and a moderate amount of lactic acid only.

Studies with isolates grown in glucose-enriched and glucose-free media. GLC was performed on glucose-enriched and glucose-free cultures of 55 saccharolytic, subgingival crevice isolates that included representative strains of B. melaninogenicus, B. oralis, Fusobacterium spp. and L. buccalis. Table 21 shows the median values and the range of results obtained with 49 isolates tested in glucose-enriched media (PPYG; PPYSG) and 38 isolates tested in glucose-free media (PPY; $\frac{1}{2}PPY_{12}$). A total of 32 isolates were tested in both glucose-enriched and glucose-free media. The volatile fatty acid profiles of the 49 saccharolytic strains in the B. melaninogenicus-B. oralis group were similar to the profiles of the four saccharolytic reference strains. B. melaninogenicus ss. intermedius strains tended to produce slightly more acetic acid than the ss. melaninogenicus strains, but there was considerable overlapping in the range of results. The ranges for the other acids produced by these two

Table 21: GLC analysis on 55 subgingival isolates in glu^- and glu^+ media

Test species or subspecies	No. of strains	Medium	No. of analyses	Median concentration and range of the stated acid* (µmols per ml)				
				A	P	iB	B	iV
B. melaninogenicus ss. intermedius	20	Glu^-	17	18 (5 - 28)	<1 (0 - 1)	<1 (0 - 2)	0	3 (1 - 9)
		Glu^+	16	20 (5 - 25)	1 (0 - 5)	<1 (0 - 1)	0	<1 (0 - 3)
B. melaninogenicus ss. melaninogenicus	22	Glu^-	22	9 (5 - 15)	<1 (0 - 2)	<1 (0 - 1)	0	2 (0 - 4)
		Glu^+	25	15 (5 - 27)	<1 (0 - 4)	0 (0 - <1)	0	<1 (0 - 2)
B. oralis	7	Glu^-	3	14 (9 - 23)	<1 (0 - 1)	0 (0 - <1)	0	1 (0 - 4)
		Glu^+	12	14 (8 - 26)	0 (0 - 2)	0	0	0 (0 - <1)
Fusobacterium sp.	2	Glu^-	2	14	2	0	14	0
		Glu^+	2	11	2	0	14	0
Leptotrichia buccalis	4	Glu^-	1	9	0	0	0	0
		Glu^+	4	10 (4 - 20)	0 (0 - <1)	0	0	0

* see footnote table 18.

subspecies also overlapped; the different median values may reflect population differences, but individual strains could not be reliably distinguished. Similarly, B. oralis strains could not be distinguished from strains of B. melaninogenicus ss. melaninogenicus on the basis of the volatile fatty acids produced in these media. Propionic acid was a minor product of most strains. The reduction in iso-valeric and iso-butyric acid production in glucose enriched media correlated with increased growth in these media. n-Butyric acid was not produced by any of these strains. The two Fusobacterium and four Leptotrichia isolates gave volatile fatty acid profiles that were distinct from each other and consistent with the profiles of the respective reference strains. The Leptotrichia strains produced more acetic and succinic acid than the reference strain; two of the isolates produced traces of propionic acid in PPYG medium.

Quantitative lactic and succinic acid estimations were performed for 29 of the B. melaninogenicus and B. oralis strains (table 22). The lactic acid concentrations were consistently reduced four-fold in glucose enriched media with 21 strains tested in both glucose enriched and glucose free media. B. melaninogenicus ss. intermedius isolates produced less lactic acid (≤ 25 µmols/ml) than was produced by the reference strain NCTC 9336 (see below). B. oralis strains produced less lactic acid than B. melaninogenicus strains, but the ranges obtained in both glucose enriched

Table 22: Production of lactic and succinic acids by 29 subgingival isolates of B. melaninogenicus and B. oralis in glu^+ and glu^- media

Test species or subspecies	Medium	No. of analyses	Median value and range of the stated acid (μmols per ml)	
			Lactic	Succinic
B. melaninogenicus ss. intermedius	glu^-	10	75 (30-100)	26 (6-52)
	glu^+	5	12 (5-25)	19 (10-38)
B. melaninogenicus ss. melaninogenicus	glu^-	13	57 (23-91)	38 (10-74)
	glu^+	15	8 (2-33)	21 (12-40)
B. oralis	glu^-	2	20 & 33	16 & 17
	glu^+	11	5 (0-15)	24 (10-40)

and glucose free media overlapped. Five B. oralis strains failed to produce lactic acid in glucose enriched media, but four strains produced 10 µmols or more per ml.

Clinical isolates grown in fully supplemented medium. As a result of the initial studies, PPYSG medium was selected for GLC analysis of the fatty acids produced by 42 clinical isolates of Bacteroides spp. Twelve asaccharolytic strains were tested after incubation for 7 days; all other isolates were tested after 2 days. The only B. fragilis isolate tested produced major amounts of succinic and acetic acids and a trace of propionic acid; two B. ovatus isolates produced similar profiles but one isolate also produced 5 µmols/ml of lactic acid. One strain identified as B. corrodens produced a similar range of acids to asaccharolytic B. melaninogenicus strains; acetic and lactic acids were the major products with propionic, iso-butyric, n-butyric and iso-valeric acids as minor products; succinic acid was not produced. Seventeen strains of B. melaninogenicus ss. asaccharolyticus produced typical profiles; lactic (27-150 µmols/ml) and acetic (5-27 µmols/ml) acids were the major products and n-butyric acid was produced in moderate amounts (5-13 µmols/ml); seven strains did not produce succinic acid but five strains produced $\geq$ 10 µmols/ml (10-19 µmols/ml); propionic and iso-valeric acids were minor products.

The saccharolytic strains of B. melaninogenicus studied in this section included eight faecal, two subgingival and

eight high vaginal swab (HVS) isolates. All 18 strains (10 ss. intermedius; 8 ss. melaninogenicus) produced similar volatile fatty acid profiles. The median values and the ranges of the acids produced were virtually identical to those reported for the same subspecies in glucose enriched media in table 21. Lactic and succinic acid results differed between the two subspecies on this occasion and also differed from the results reported in table 22.

The range, median and mean values obtained from faecal, subgingival and HVS isolates are compared in table 23. The values reported from the subgingival isolates include the results on strains included in table 22 and on nine strains of B. melaninogenicus ss. melaninogenicus from the collaborative survey (see below).

Subgingival strains of B. melaninogenicus ss. intermedius produced lactic and succinic acids in similar quantities to faecal strains of the same subspecies. Eight of 11 subgingival and faecal strains consistently produced <30 µmols/ml of lactic acid and seven of 11 strains produced <40 µmols/ml of succinic acid. Two faecal and one subgingival strain produced >45 µmols/ml of lactic acid. HVS strains of B. melaninogenicus ss. intermedius produced more lactic and succinic acids than the subgingival or faecal strains. Three of four HVS strains consistently produced >60 µmols/ml

Table 23: Comparison of lactic and succinic acid production by saccharolytic strains of B. melaninogenicus from different clinical sites

Test subspecies	Source	No. of strains	No. of analyses	Range of the stated acid* (μmols per ml)		Median	Mean
	Subgingival	6	10	L	6 - 46	20	20.5
				S	10 - 81	38	39.5
B. melaninogenicus ss. intermedius	Faecal	5	7	L	10 - 52	25	28
				S	9 - 71	40	37
	HVS Ø	4	7	L	34 - 110	65	74
				S	24 - 85	68	65
	Subgingival	17 §§	17	L	2 - 33	8	9
				S	12 - 40	19	23
B. melaninogenicus ss. melaninogenicus	Faecal	3	3	L	4 - 38	20	20.6
				S	11 - 69	46	42
	HVS	4	4	L	10 - 55	33	33.5
				S	35 - 150	83	95

* L = Lactic; S = Succinic

All analyses on 2-day cultures in glu$^+$ medium

Ø HVS = high vaginal swab isolates

§§ Includes our strains analysed in collaborative survey

of lactic acid and the other strain produced >60 μmols/ml of the acid in one of two cultures tested. All 4 HVS strains produced >66 μmols/ml of succinic acid although 2 of 4 strains only did so in one of two cultures tested. All analyses were performed on 2-day cultures in PPYSG medium and possible reasons for the culture-to-culture variation observed with a few strains were not elucidated.

Subgingival and faecal strains of B. melaninogenicus ss. melaninogenicus produced similar quantities of lactic and succinic acids. Seventeen of 20 strains produced < 13 μmols/ml of lactic acid and 18 of 20 strains produced < 40 μmols/ml of succinic acid. The high median value of 46 μmols/ml for succinic acid production by faecal strains in table 23 is not significant where only three strains were studied. Three of four HVS strains of B. melaninogenicus ss. melaninogenicus produced >30 μmols/ml of lactic acid and >80 μmols/ml of succinic acid.

Three additional strains were provisionally identified as B. melaninogenicus ss. intermedius on the basis of indole production, gelatin digestion and the slow, weak fermentation of glucose. These isolates, however, produced n-butyric acid on two occasions in PPYSG medium; two also failed to produce succinic acid and were therefore more typical of asaccharolytic strains, but the third isolate produced 18 μmols/ml of this acid.

Collaborative studies with B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus. Forty strains belonging to these groups were tested as part of a collaborative investigation instigated by the I.C.S.B. Taxonomic Sub-committee on Gram-negative anaerobic rods. Twelve strains were referred by the sub-committee, six strains were referred by other colleagues and 22 strains were isolated from subgingival plaque. Table 24 shows the range of fatty acids produced by the strains. Loesche's strain 7CM designated B. oralis has been excluded from the B. oralis group in the table as it is more typical of a B. fragilis strain (see Holbrook, Duerden and Deacon, 1977); similarly the referred strains ATCC 15930, VPI 7570A and no. 30 were found to produce black-pigmented colonies on lysed blood agar and were included in the B. melaninogenicus ss. melaninogenicus group.

Table 25 shows population differences between the groups represented in the study. The individual results were assigned to four grades that appear to represent significant differences between strains. Individual strains of B. melaninogenicus ss. melaninogenicus could not be differentiated from strains of B. oralis on the basis of GLC results alone. With one exception, the median values and ranges of the acids produced by B. melaninogenicus ss. melaninogenicus and B. oralis strains were similar to the values obtained previously (see table 21) and overlapped

Table 24: Fatty acids produced by strains in the collaborative study in PPYSG medium

Test species or subspecies	No. of strains	Median concentration and range of the stated acid* (μmols per ml)						
		A	P	iB	B	iV	L	S
Bacteroides melaninogenicus ss. melaninogenicus	23 †	20 (10-46)	1 (0-4)	0 (0-<1)	0	<1 (0-2)	7 (0-33)	22 (14-40)
Bacteroides oralis	10	15 (4-26)	<1 (0-2)	0	0	0 (0-<1)	0 (0-10)	21 (11-36)
Bacteroides ochraceus	6	10 (3-14)	0 (0-2)	0	0	0 (0-<1)	0 (0-5)	19 (11-31)
Loesches strain 7CM	1 ø	22	<1	0	0	0	0	45

* See footnote to table 18

† includes ATCC 15930, VPI 7570A and 30 see Results and Holbrook & Duerden, 1974.

ø not included in B. oralis. See text.

Table 25: The production of volatile fatty acids by the 40 strains examined in the collaborative study

Fatty acid	Concentration value	Percentage of the test organisms* producing the stated amount of acid		
		B. melaninogenicus ss. melaninogenicus (23)	B. oralis (11)	B. ochraceus (6)
Acetic	+	0	18.2	66.7
	++	100	81.8	33.3
Propionic	-	13	36.4	50
	tr	34.8	36.4	16.7
	+	52.2	27.2	33.3
iso-Butyric	-	82.6	100	100
	tr	17.4	0	0
n-Butyric	-	100	100	100
iso-Valeric	-	21.7	63.6	83.3
	tr	69.5	36.4	16.7
	+	8.7	0	0
n-Valeric	-	100	100	100
Lactic	-	8.7	63.6	83.3
	tr	69.6	18.2	16.7
	+	17.4	18.2	0
	++	4.3	0	0
Succinic	+	39.1	45.5	50
	++	60.9	54.5	50

* No. tested shown in brackets

Volatile acids:

Concentration value	µmols per ml:	
> 10	"	++
1.1 - 10	"	+
0.2 - 1.0	"	tr (trace)
< 0.2	"	-

Lactic and Succinic acids:

Concentration value	µmols per ml:	
> 20	"	++
10 - 20	"	+
2 - 10	"	tr
< 2.0	"	-

with results obtained with the B. ochraceus strains. However, B. ochraceus strains generally produced smaller quantities of acetic and lactic acids. Three referred strains of B. melaninogenicus ss. melaninogenicus (WAL 2724, WAL 2721 and VPI 7570A) produced > 28 umols/ml of acetic acid.

A study of culture-to-culture variation. Despite careful attention to detail, particularly with regard to size and condition of inoculum, incubation time, sample size at injection and the daily use of appropriate standards and controls, variation occurred in the quantitative results obtained with different cultures of the same strains. The results of analyses on two separate two-day cultures of each of 3 reference strains in the same medium are shown in table 26. In all analyses the major products of each strain showed small variations but were always present in significant amounts. Minor products varied in concentration but were consistently detected; trace products were occasionally absent.

Note: Some of the results presented in this section were incorporated in a report to the I.C.S.B. Taxonomic Sub-committee on Gram-negative anaerobic rods, submitted by members of the Microbial Pathogenicity Research Laboratory (MPRL) team under Professor J.G. Collee.

Table 26: The quantitative variation in fatty acid production by three reference strains of Bacteroides analysed in PPYSG medium on two separate occasions

Species or subspecies	Strain No.	Study*	Approximate concentration of the stated acid						
			A	P	iB	B	iV	L	S
B. oralis	NP 333	I	19	2	0	0	1	3	36
	NP 333	II	10	1	0	0	0	0	22
B. melaninogenicus ss. melaninogenicus	ATCC 15930	I	20	1	0	0	0	0	25
	ATCC 15930	II	14	0	0	0	0	0	27
B. melaninogenicus ss. melaninogenicus	VPI 4196	I	26	2	1	0	1	12	38
	VPI 4196	II	20	1	1	0	1	6	39

* I = Collaborative study
II = Media comparison study
} All analyses performed on 2-day cultures.

see footnote table 18.

The report is bound in this thesis in Appendix III; tables relevant to the report are placed in the pocket inside the back cover. Further results have been published in a paper written in collaboration with Dr B.I. Duerden and Dr W.P. Holbrook. This paper is bound in Appendix IV. A further paper based on these results, also written in collaboration with Drs Duerden and Holbrook, has been accepted for publication in the Journal of Medical Microbiology. The title page and summary are bound into Appendix IV. Documentary evidence of acceptance is presented with this thesis.

Characterisation of additional strains of Bacteroidaceae

The 15 strains listed in table 8 were studied by 5 approaches including morphology, biochemical tests, tolerance tests, antibiotic resistance tests and GLC of the fatty acid metabolic products of their fermentation in PPYSG medium.

Morphology

All were Gram-negative, non-sporing, obligately anaerobic rods or cocco-bacilli. Pleomorphism was common and the microscopic appearances varied. Cellular morphology was not of discriminatory value. The 6 referred

strains thought to be B. oralis were all similar ranging from Gram-negative cocco-bacilli to short or medium bacillary forms occurring singly, in pairs or short chains with longer, occasionally bizarre filaments produced in 2-day cultures on BA or in Robertson's CMB medium. Colonies on BA after incubation for 2 days were 0.5 to 1.0 mm in diameter, round or irregular, convex, opaque, light grey, mostly shiny and either non-haemolytic or incompletely haemolytic.

Strain VPI 3300, a proposed representative of a new subspecies of B. melaninogenicus (B. melaninogenicus ss. levii), was similar to the putative B. oralis strains occurring as mainly Gram-negative cocco-bacilli in pairs and chains with occasional longer filaments. After culture for 2 days on BA there were non-pigmented pinpoint colonies; black-pigmented colonies developed on lysed BA after incubation for 7 days, but not before that time.

On BA at 7 days, the strain produced non-pigmented 3.0-mm round, high convex, opaque, moist, shiny, light grey, non-haemolytic colonies. Discrete colonies on BA at 7 days showed a dark grey-brown edge.

The 2 strains designated B. disiens, VPI 8057 and 7852 were also Gram-negative and pleomorphic ranging from

coccal forms to short bacilli occurring singly, in pairs or clusters.

On BA the colonies at 2 days were similar to the B. oralis strains and showed incomplete haemolysis. The 2 strains designated B. bivius showed the same degree of pleomorphism and similar colonial morphology.

The strains designated B. splanchnicus, NCTC nos. 10825 and 10826 were also pleomorphic and ranged from coccal forms to medium-sized bacilli and longer filaments. Two colony variants were observed on the BA plates of both strains after incubation for 2 days: 1) a 1 to 2-mm, round, convex colony and a low-convex, plateau or draughtsman-like colony of similar size. Both colonies were opaque, matt or shiny, light grey and β-haemolytic.

The clinical isolate Gnab 55s produced Gram-negative short stout rods with rounded ends in Robertson's CMB medium and also on BA at 2 days. Many of the cells taken from the colonies on BA had a central unstained area and some very large rods were present. On BA at 2 days, typical non-haemolytic bacteroides colonies were seen and by 7 days they had developed light grey centres with darker grey at the edges and remained non-haemolytic.

Results of tolerance, antibiotic resistance tests and GLC analyses of the fatty acid products

The results of the tolerance, antibiotic resistance and GLC analyses of the fatty acid end-products are given in table 27. Penicillin sensitive and penicillin resistant variants were isolated from the primary cultures of the B. splanchnicus strain NCTC 10825. Both variants were tested independently. The type strain of B. ruminicola ss. brevis, GA 33 failed to grow in any of the media used in the characterisation scheme and is not included.

All the putative B. oralis strains were inhibited by the bile salts. All except one strain were inhibited by the four dyes. The strain WAL 3281 was not inhibited by Victoria blue 4R but was inhibited by ethyl violet, gentian violet and brilliant green. The B. melaninogenicus ss. levii strain VPI 3300 was inhibited by all the dyes and bile salts, as were the strains of B. bivius and B. disiens. The B. splanchnicus strains NCTC 10825 (both variants) and 10826 were able to grow in the presence of taurocholate 0.5% and the dye Victoria blue 4R. They were inhibited by deoxycholate 0.1% and by the dyes gentian violet and brilliant green. Strain NCTC 10826, and the

Table 27. The results of tolerance and antibiotic resistance tests and the GLC analysis of the fatty acid end-products of fermentation for the additional strains of Bacteroides spp. studied

Test	Pattern of results* obtained from the test strains														
	VPI 8906D	VPI 9958	VPI 5540	VPI 7880	WAL 3030	WAL 3281	VPI 3300	Gnab 55s	NCTC 10825^{S} †	NCTC 10825^{R} †	NCTC 10826	VPI 6822	VPI 6318	VPI 7852	VPI 8057
Tolerance tests:															
taurocholate 0.5% (T)	I	I	I	I	I	I	I	+	+	+	I	I	I	I	I
deoxycholate 0.1% (D)	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
T + D	I	I	I	I	I	I	I	+	I	+	I	I	I	I	I
Victoria blue 4R	I	I	I	I	I	+	I	+	+	+	+	I	I	I	I
Ethyl violet	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Gentian violet	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Brilliant green	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I
Antibiotic resistance:															
Metronidazole 5µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin 1000µg	S	R	S	R	R	R	R	R	R	R	R	R	R	R	R
Kanamycin 1000µg	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Penicillin 1.5u	S	S	R	S	S	S	S	R	S	R	R	R	S	R	S
Rifampicin 15µg	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Clindamycin 2µg	S	S	S	S	S	S	S	R	S	S	S	S	S	S	S
GLC analysisø **Fatty acids produced:**															
Acetic	++	+	++	++	++	+	++	++	++	++	++	++	++	++	++
Propionic	+	tr	tr	+	tr	tr	+	+	+	+	+	+	tr	tr	+
isoButyric	-	-	tr	tr	-	tr	+	tr	+	+	+	tr	tr	tr	-
nButyric	-	-	-	-	-	-	++	-	++	++	+	-	-	-	-
isoValeric	tr	-	tr	tr	tr	tr	+	+	+	+	+	tr	tr	tr	tr
Lactic	tr	-	++	++	+	tr	++	++	++	++	++	++	++	+	+
Succinic	++	+	++	++	++	++	-	++	++	++	++	++	++	++	++

* + = positive result - = negative result I = inhibited R = resistant S = sensitive tr = trace only

† Strain NCTC 10825 yielded penicillin sensitive(S) and penicillin resistant(R) variants

ø For concentration ranges see footnote table 25.

penicillin sensitive variant of NCTC 10825, were not inhibited by the combined bile salts but the penicillin resistant variant of NCTC 10825 was inhibited. The penicillin sensitive variant and NCTC 10826 showed poor growth in the presence of ethyl violet; the other variant was inhibited by this dye.

All the test strains were sensitive to metronidazole (5 µg) and rifampicin (15 µg) and all were resistant to kanamycin 1000 µg. The clinical isolate Gnab 55s alone was resistant to clindamycin. Four of the B. oralis strains were resistant to neomycin 1000 µg. Strain VPI 8906D and VPI 5540 were sensitive to neomycin (1000 µg). All the other test strains were resistant to this antibiotic. Strain VPI 5540 was the only B. oralis strain sensitive to penicillin, 1.5 units. The B. splanchnicus strain NCTC 10826, one strain of B. disiens, VPI 7852 and one strain of B. bivius, VPI 6822 were also resistant to penicillin.

All the test strains produced acetic and propionic acids in PPYSG medium. The B. melaninogenicus ss. levii strain, VPI 3300 alone failed to produce succinic acid. The major products of most strains were succinic, acetic and lactic acids. Only one strain of B. oralis, VPI 9958

failed to produce lactic acid. Four strains, the B. splanchnicus strain, NCTC 10826, both variants of NCTC 10825 and the B. melaninogenicus ss. levii strain, VPI 3300 produced >140 µmols/ml of lactic acid; the latter producing >280 µmols/ml. Iso-butyric and iso-valeric acids were minor products of most strains. Significant quantities of n-butyric acid were produced by the B. melaninogenicus ss. levii strain, VPI 3300 and by the 3 B. splanchnicus strains. No other strain produced this acid.

Results of biochemical tests

The results of these tests are shown in table 28. None of the strains produced oxidase and only the penicillin resistant variant of B. splanchnicus, NCTC 10825 produced catalase. Only the B. oralis strain, VPI 9958 failed to produce hydrogen sulphide. None of the strains produced lipase, reduced nitrate or were stimulated by 20% ox bile. The 3 strains of B. splanchnicus produced indole but all other test strains did not. The B. splanchnicus strain NCTC 10826 and the clinical isolate Gnab 55s failed to digest gelatin; all the other strains did so although the B. oralis strain, VPI 8906D, was only weakly positive. Some strains were weakly haemolytic while others were non-haemolytic. Only one B. oralis

Table 28. Biochemical reactions of the additional strains of Bacteroides spp. studied

Test	Pattern of results* obtained from the test strains														
	VPI 8906D	VPI 9958	VPI 5540	VPI 7880	WAL 3030	WAL 3281	VPI 3300	Gnab 55s	NCTC 10825^{S} †	NCTC 10825^{R} †	NCTC 10826	VPI 6822	VPI 6318	VPI 7852	VPI 8057
Growth in air + CO_2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pigment production	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Haemolysis	±	-	+	±	-	-	-	-	+	+	+	-	-	±	±
H_2S production	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase production	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Aesculin hydrolysis	+L	+	-	-	+	+	-	+	-	-	+	-	-	-	-
Gelatin digestion	±	+	+	+	+	+	+	-	+	+	-	+	+	+	+
Lipase production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-
Bile stimulation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Fermentation of:															
Glucose	+	+	+	+	+	+	+	+	+	+	+	+L	+L	+	+
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Maltose	+	+	+	+	+	+	-	+	+L	-	-	+	+	+	+
Sucrose	+	+	-	-	+	+	-	+	-	-	-	-	-	-	-
Rhamnose	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+L	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	+	-	-	+L	-	-	-	-
Dextran hydrolysis	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

* + = positive result - = negative result +L = late positive ± or ⊥ = weak positive

† Strain NCTC 10825 produce penicillin sensitive (S) and penicillin resistant (R) variants.

strain, VPI 5540, and all 3 B. splanchnicus strains produced β haemolysis after incubation for 7 days. All the strains fermented glucose but the two strains of B. bivius, VPI nos. 6822 and 6318 were late. Two B. oralis strains, VPI 7880 and 5540 and all the B. bivius and B. disiens strains produced a significant fall in pH to ≤ 6.5 in glucose-free thioglycollate medium within 2 days. All the other test strains produced little if any change in this time, remaining at or above pH 6.7. All of the test strains except the B. splanchnicus strains were acidogenic in the glucose-free PPYS medium used in the GLC analyses, many producing pH falls of almost 1.0 unit in 2 days. None of the test strains fermented mannitol, and Gnab 55s alone fermented trehalose. This strain and one B. oralis, VPI 9958 fermented rhamnose. Gnab 55s also fermented arabinose and xylose. No other strain fermented both these sugars. One B. oralis strain, VPI 8906 D was a late fermenter of arabinose and the B. splanchnicus strain NCTC 10826 was a late fermenter of xylose. Both B. disiens strains, VPI 7852 and 8057, were the only test strains that failed to ferment lactose. The B. splanchnicus strains NCTC 10825R and 10826 and the B. melaninogenicus ss. levii strain VPI 3300 failed to ferment maltose, whilst all other test strains did so. Dextran was hydrolysed by only one strain, Gnab 55s.

The indole-nitrite medium (BBL) was found to be a significant improvement on the thioglycollate medium previously used for testing nitrate reduction. The positive control strain of E. coli, NCTC 10418 and the negative control B. fragilis, NCTC 9344 grew well within 48 h and gave consistently reproducible, clear results. There were occasional growth failures with demanding test strains and others took 3-4 days to produce sufficient growth for reliable testing.

The bile stimulation test also produced consistent, clear results with the control strains used. The positive controls B. fragilis NCTC 9344 and the negative control B. melaninogenicus ss. intermedius NCTC 9338 grew rapidly in the PPY or PPYG media without added bile. The B. fragilis strain was consistently stimulated by the addition of ox bile to the PPYG medium and produced an extremely dense turbidity after overnight incubation. The turbidity in this medium was produced by a combination of maximal growth rates to high levels and a heavy crystalline deposit. No crystalline deposit occurred in PPYG medium without added bile, in PPY medium with or without bile or in the bile-containing cultures of unstimulated test strains.

Problems in fermentation testing examined with selected strains of B. melaninogenicus

In establishing the fermentation tests, Duerden et al. (1976) decided to use thioglycollate medium as the basal medium and extensive preliminary trials indicated that the acceptance of a pH difference of 0.5 of a unit or greater between the carbohydrate-containing test medium and the sugar-free control medium gave results consistent with the reported findings of other workers using the same type culture or reference strains. Testing of freshly isolated clinical strains mainly from the B. fragilis group did not present any real difficulties with this scheme but when larger numbers of oral, faecal and vaginal isolates of B. melaninogenicus were examined there were difficulties with occasional strains in the interpretation of fermentation test results. The use of a wide range of other characterisation tests, including GLC analyses of the fatty acid end-products of fermentation, allowed confident identification of some of the strains to subspecies level but a few problems still remained. Table 29 shows the discrepancies found in glucose fermentation test results during characterisation studies with some freshly isolated strains of B. melaninogenicus. The test results with thioglycollate medium in some cases

Table 29. The difference in pH between glucose-free and glucose-enriched cultures of selected freshly isolated strains of B. melaninogenicus after given periods of incubation

Test strains	The difference in pH in the test media* at a given incubation time (days)					
	Original result		Result on retesting			
	Thio	PPYS	Thio	PPYS	CMB	Provisional identification$^{\emptyset}$
WPH 57	0.1 (7)†	0.1 (7)	...	...	...	B. melaninogenicus ss. asaccharolyticus
" 98	1.1 (7)	0.3 (7)	0.2 (4)	0.3 (4)	0.1 (4)	" " " "
" 118	0.1 (7)	0.1 (7)	0.1 (7)	...	...	" " " "
" 201	1.4 (7)	1.2 (7)	1.2 (8)	0.6 (8)	1.2 (8)	" " ss. N.D.
" 202	0.3 (7)	0.6[a] (2)	0.25 (8)	0.7[a] (8)	0.6[a] (8)	" " ss. intermedius
" 210	0.1 (7)	1.2 (2)	0.1 (7)	0.8 (2)	...	" " " "
" 222	0.9 (7)	0.1 (7)	0.8 (8)	0.3 (8)	1.0 (8)	" " ss. N.D.
" 223	0.8 (7)	0.3 (7)	0.5 (8)	0.6 (8)	0.2 (8)	" " " "

Table 29. CONTD.

Test strains	The difference in pH in the test media* at a given incubation time (days)					
	Original result		Result on retesting			
	Thio	PPYS	Thio	PPYS	CMB	Provisional identification[ø]
WPH 225	0.1 (7)	1.05 (7)	...	...	...	B. melaninogenicus ss. asaccharolyticus
" 226	1.4 (7)	0.6 (7)	0.1 (4)	0.00 (4)	0.00 (4)	" " ss. N.D.
" 228	0.1 (7)	0.55[a] (7)	0.1 (8)	0.6[a] (8)	0.4[a] (8)	" " ss. N.D. ? intermedius
" 229	0.2 (7)	0.2 (7)	c	0.55[a] (8)	0.5[a] (8)	" " ss. " "
" 234	0.4 (7)	0.85 (7)	...	...	...	" " ss. N.D. ? asaccharolyticus

* Thio = Thioglycollate medium
PPYS = PPYS medium
CMB = Robertson's CMB medium
† the period of incubation (days) is given in brackets
ø identifications based on all results obtained in the characterisation tests
a = acidogenic, the pH in glucose-free medium was ≤ 6.2
c = contaminated
N.D. = not definite
... = not tested

showed marked differences from the results obtained in the PPYS and PPYSG cultures prepared for GLC analyses. The table also shows the results of re-testing in the same media and in Robertson's CMB medium. All the strains listed in the table produced indole and digested gelatin but did not hydrolyse aesculin. Their antibiotic resistance patterns were identical and similarly their bile salt and dye tolerance test results were of no discriminatory value.

The WPH strains 57, 98 and 118 had all produced n-butyric acid in PPYSG medium and appeared to be typical B. melaninogenicus ss. asaccharolyticus strains. The strain WPH 98 fermented glucose on one occasion in thioglycollate medium after incubation for 7 days, but both on initial testing and subsequent testing it failed to ferment after incubation for 2 and 4 days respectively.

The WPH strains 201, 222, 223 and 226 fermented glucose in thioglycollate medium and produced n-butyric acid in PPYSG medium. At the time of first testing, it was thought that these strains might possibly represent a new subspecies of B. melaninogenicus (see Holbrook, Duerden and Deacon, 1977) and required further study.

The WPH strains 202, 210, 228, 229 and 234 all failed to ferment glucose in thioglycollate medium on

first testing, however strains 202, 210 and 228 failed to produce n-butyric acid in PPYSG medium and had shown fermentation of glucose in this medium. They were provisionally identified as B. melaninogenicus ss. intermedius but required further study. On re-testing, the previous results in thioglycollate and PPYSG media were confirmed with only minor variations in the pH differences observed. The results for strains 202 and 228 grown in Robertson's CMB medium were similar to those obtained with PPYSG medium.

The WPH strain 229 failed to ferment glucose in either thioglycollate, after incubation for 7 days, or in PPYSG medium after 2 days. It produced 4+ growth in the PPYSG medium but had not produced n-butyric acid. It was re-tested in PPYSG and glucose-enriched CMB medium and after 8 days it produced pH differences of 0.55 and 0.5 of a unit respectively between the glucose-free controls and the test media. It was acidogenic in both media. The final pH was 5.2 ± 0.05 in both glucose-enriched media. The strain was provisionally identified as B. melaninogenicus ss. intermedius, but it required further investigation.

The WPH strains 225 and 234 failed to ferment glucose after 7 days in thioglycollate medium but had fermented

glucose in PPYSG medium in the same time. They produced n-butyric acid in the PPYSG medium and were identified as strains of B. melaninogenicus ss. asaccharolyticus.

In general the freshly isolated strains grew better in the PPYS or PPYSG media but there was an increased incidence of substantial pH falls occurring in the glucose-free PPYS medium. It was considered that the discrepancies encountered in the fermentation tests may have been in part due to the presence of serum in PPYS and PPYSG media; however the results in Robertson's CMB medium were also at variance with the results on thioglycollate medium. It appeared that some isolates were unable to grow, or grew poorly in the basal thioglycollate medium and their growth was not stimulated by addition of carbohydrate.

Further investigations were undertaken in thioglycollate medium and in $\frac{1}{2}$PPY medium not supplemented with horse serum. Table 30 shows the results obtained from fermentation and glucose utilisation studies obtained from 5 reference strains and 13 clinical isolates.

The five reference strains produced consistent results in all 3 fermentation test media. The growth of the strains fermenting glucose or maltose were stimulated

Table 30. Results of growth, fermentation and glucose utilisation studies on five reference and thirteen clinical isolates of B. melaninogenicus.

Test Strain:		NCTC 9337	VPI 4196	ATCC 15930	NCTC 9336	VPI 3300	WPH 57	98	118	201	202	210	214	222	223	226	228	229	234
Growth stimulated by glucose * in	thio	-ℓ	+	+	+	+	-	-	-ℓ	⊥	-	-	-ℓ	+	+	+	-	-	-
	½ PPYG	-ℓ	+	+	+	+	-	-	-	-	-	-	-	-	+	⊥	-	-	-
Fermentation † of glucose in ½ PPYG	1 day	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2 "	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4 "	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	7 "	-	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-
Glucose utilization in ½ PPYG (%)	1 day	4.0	21.5	6.0	18.4	5.9	5.3	7.6	5.7	6.8	7.0	5.5	5.5	5.3	9.3	10.0	5.1	5.6	2.7
	2 "	5.2	27.0	12.4	32.8	Nil	Nil	2.2	2.2	6.4	0.3	0.3	1.9	1.1	1.5	Nil	1.7	Nil	2.3
	4 "	8.7	29.1	25	39.0	15.9	8.9	7.6	6.6	7.3	7.5	10.8	10.1	11.2	12.4	7.9	9.0	8.5	8.7
	7 "	10.8	29.4	23.2	39.0	17.3	8.5	8.5	6.1	11.6	2.9	6.8	2.9	7.0	17.1	15.3	6.6	7.7	5.4
n Butyric acid ϕ production		+	-	-	-	+	+	+	+	+	-	-	+	+	+	+	-	-	+
Fermentation † of glucose in thio	2 days	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	7 "	-	+	+	+	+	-	-	-	+	-	-	+	+	+	+	-	-	-
Growth in thio stimulated by maltose		-ℓ	⊥	⊥	+	⊥	-	-	-	-ℓ	-	-	-ℓ	-	⊥	⊥	+	⊥	-
Fermentation † of maltose in thio	2 days	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7 "	...	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-

* Assessed by visual turbidity differences after incubation for up to 7 days

† pH difference of ≥ 0.5 unit between glucose free and glucose enriched medium

ϕ determined by GC analysis on cultures in PPYSG medium

ℓ lysis in carbohydrate enriched medium after 7 days.

... not tested

\+ = positive

⊥ = weak positive

\- = negative

thio = thioglycollate medium

by addition of the carbohydrate to the test media. The B. melaninogenicus ss. asaccharolyticus strain NCTC 9337 was not stimulated by addition of either carbohydrate and there was evidence of cell lysis occurring after incubation for 7 days. Glucose was slowly utilised by this strain, reaching a maximum of 10.8% after 7 days. The B. melaninogenicus ss. melaninogenicus strain VPI 4196, the ATCC strain 15930 and the ss. intermedius strain NCTC 9336 fermented glucose in all the test media and fermented maltose in thioglycollate medium. All 3 strains utilised glucose at significantly higher levels than the B. melaninogenicus ss. asaccharolyticus type strain NCTC 9337. Maximum utilisation had taken place by 4 days and incubation for 7 days produced little or no change to the level of utilisation. The B. melaninogenicus ss. levii strain VPI 3300 was a slow fermenting strain but it did not present any difficulties in the interpretation of its results. It had not fermented glucose after 2 days in $\frac{1}{2}$PPYG medium but had fermented by 4 days, producing an average pH difference of 0.8 of a unit between $\frac{1}{2}$PPY and $\frac{1}{2}$PPYG media; at 7 days the difference was 1.2 units. In thioglycollate medium it was also negative at 2 days but fermentation had clearly taken place by 7 days. It did not ferment maltose in this medium and in this respect differed from the other 3 saccharolytic reference strains.

It utilised glucose to levels intermediate between the ss. asaccharolyticus strain and the 3 saccharolytic test strains, VPI 4196, ATCC 15930 and NCTC 9336. Maximum utilisation occurred after 4 to 7 days; the results on these two days were not significantly different. Like the B. melaninogenicus ss. asaccharolyticus strain NCTC 9337, it produced n-butyric acid in PPYSG medium but in contrast it was stimulated to better growth by addition of glucose to the test media.

The earlier results of WPH strains 57, 98 and 118 were confirmed on this occasion. WPH 98 also did not ferment in either thioglycollate or ½PPYG media after incubation for 7 days. All 3 strains were confidently identified as strains of B. melaninogenicus ss. asaccharolyticus.

The WPH strains 201, 214, 222, 223 and 226 again fermented glucose in thioglycollate medium but only strain 223 fermented this substrate in ½PPYG medium. This strain utilised more glucose in ½PPYG medium finally reaching levels similar to that achieved by the reference strain of B. melaninogenicus ss. levii. Apart from the absence of fermentation and the low utilisation figures in ½PPYG medium the other strains, 201, 214, 222 and 226 produced a pattern of results similar to those obtained with the same reference strain. Three of the strains, WPH 222,

223 and 226 utilised slightly more glucose in ½PPYG medium than the reference strain of B. melaninogenicus ss. asaccharolyticus, but the differences were not considered sufficient to allow certain differentiation.

The identity of the WPH strains 202, 210, 228 and 229 remains in doubt. They were previously thought to be strains of B. melaninogenicus ss. intermedius, but the earlier discrepancies in their characterisation test results were confirmed. On this occasion they again failed to ferment glucose in thioglycollate medium and this result was confirmed by the absence of fermentation and low levels of glucose utilisation found after 7 days in ½PPYG medium. The strains were however not typical of B. melaninogenicus ss. asaccharolyticus because n-butyric acid was not produced in PPYSG medium even after prolonged incubation for 7 days. In addition the strain WPH 228 fermented maltose in thioglycollate medium on this occasion for the first time.

The WPH strain 229 did not ferment glucose in either test medium on this occasion and with low glucose utilisation figures it appeared to be a typical B. melaninogenicus ss. asaccharolyticus except for the absence of n-butyric acid production as noted on first testing. This result was subsequently confirmed on retesting after incubation for 7 days.

In contrast to its earlier results in PPYSG medium the WPH strain 234 failed to ferment glucose in serum free ½PPYG medium and produced a typical B. melaninogenicus ss. asaccharolyticus pattern confirming the original characterisation scheme results.

The agreement in pH readings between duplicate cultures in ½PPY or ½PPYG media was excellent. Readings for many pairs of tubes differed by only 0.05 of a unit and the maximum difference observed was 0.1 of a unit. Similarly, the agreement between pairs of glucose utilisation absorbance readings was excellent. The mean difference taken on 69 pairs of test cultures was 2.4% (0.010) and the maximum difference of 8.5% (0.036) was observed with only one pair of cultures. The procedure itself was simple and reproducible results were obtained from the uninoculated control media and the aqueous standard when tested on different occasions. The stability of the absorbance readings with time was excellent when initial colour development took place on the bench. It was found however that deterioration in the absorbance reading could occur if the sample was left in the light path during the initial colour development. In this instance a stable plateau reading was not obtained. Tests with 3 different dilutions of the glucose standard over the

expected absorbance range of the diluted test samples showed that the 100, 200 and 300 mgm/dl standards produced a linear increase in absorbance readings.

DISCUSSION

The Gram-negative anaerobic bacilli of the family Bacteroidaceae are now recognised as important members of the commensal flora of Man and other animals. Recent advances in anaerobic culture and identification of these organisms has led to an increasing awareness of the importance of some species as pathogens in many clinical conditions. Infections involving these organisms often arise endogenously and they are frequently found in mixed cultures. Prompt identification is likely to be of help in determining whether isolates present in mixed cultures are pathogens or contaminating normal flora or are merely transients not involved in the infectious process. Complete identification to species or subspecies level can assist in establishing the pathogenic potential of some species. In this way it has been possible to show that strains of B. fragilis (B. fragilis ss. fragilis) are more frequently involved in causing clinical infections than the closely related strains of B. thetaiotaomicron, B. vulgatus and B. distasonis that are more numerous in the gastrointestinal tract (Holdeman, Cato and Moore, 1974; Hill and Altemeier, 1977).

Clinical laboratories must place reliance upon simple, easily performed tests but there is a need to continue the search for more rapid and reliable means of isolation and

for improved methods of characterisation. When the present author joined the Edinburgh team it was planned to extend the characterisation scheme of Duerden *et al.* (1976) by increasing the range of tests - particularly by developing gas-liquid chromatographic procedures for the analysis of fatty acid metabolic products and by examining a wider range of reference and fresh clinical isolates of the Gram-negative, anaerobic bacilli. It was decided to re-examine some additional aspects of the anaerobic culture procedures in use and to look at the possible need for improved culture media that would satisfy the nutritional requirements of demanding fresh isolates from the genus *Bacteroides*. Accordingly this Discussion will (i) review modern anaerobic culture techniques in relation to current hypotheses on the factors involved in anaerobiosis and evaluate the results of studies on some aspects of the handling procedures used by the Edinburgh team; (ii) examine the growth of bacteroides organisms in liquid culture media using *B. melaninogenicus* ss. *asaccharolyticus* as a model; (iii) discuss the results of gas chromatographic analyses on the fatty acid metabolic products of a range of reference and fresh isolates of clinically important strains from the family Bacteroidaceae and evaluate the possible use of this approach to supplement existing diagnostic procedures in clinical laboratories; (iv) evaluate additional characterisation tests introduced into the established

scheme of Duerden et al. (1976) and discuss the results obtained from isolates that widen the discriminatory range of the scheme, and (v) consider some problems encountered in the routine fermentation testing of some clinical isolates.

The successful laboratory culture and characterisation of this group of organisms depends upon a number of factors. The fundamental physical requirements of temperature, pressure, moisture and pH are similar to those of many of the saprophytic bacteria that are adapted to living in close association with human or animal hosts and these have been discussed in various textbooks (see for instance, Cruickshank et al., 1975). Other factors including the condition of the culture media, whether stored or freshly prepared, and the condition and size of culture inocula, are also important but this thesis is mainly concerned with the methods used to handle these organisms and provide the anaerobic or appropriately reduced conditions necessary for growth and also with their nutritional requirements in complex culture media. The nutritional requirements of the group vary and are discussed in a later section.

A review of modern methods of anaerobic culture and the importance of oxygen and low redox potentials

The historical development of anaerobic culture methods and the current hypotheses on the factors involved in anaerobiosis, that form the theoretical basis of modern culture methods, were reviewed in the Introduction.

It is well known that oxygen plays a key role in preventing the growth of obligate anaerobes either by raising the redox potential (Eh) of culture media above the levels at which growth can be initiated or by virtue of its direct or indirect toxicity. The current hypotheses direct attention mainly to the interference with cellular processes resulting from oxidation of reduced enzymes, co-enzymes or growth factors, particularly those containing sulphydryl groups, and to the importance of free radicals, superoxide anions and singlet oxygen (Morris, 1975).

The earlier hypothesis that the formation of intracellular peroxides was toxic for obligate anaerobes that do not produce catalase or peroxidases (McLeod and Gordon, 1923; Gordon, Holman and McLeod, 1953) has not been entirely refuted although some workers have disputed its importance as a factor involved in anaerobiosis (Smith, 1967; Watt, 1972). Other workers have directed attention to the production of

organically derived peroxides or other products of the interaction of oxygen with the chemical constituents of a medium and they have shown that media prepared or stored under aerobic conditions may be inhibitory for some bacteria (Proom et al., 1950; Barry et al., 1956; Smith, 1967).

As a result of the increasing evidence of the toxic effects of oxygen and its derivatives at the cellular and biochemical levels (Haagaard, 1968; Morris, 1975) recent improvements in anaerobic methodology have tended to concentrate on the physical exclusion of oxygen; a notable exception has been the use of cysteine-dithiothreitol in solid media for the cultivation of Clostridium novyi type B and D (Moore, 1968; Collee et al., 1971). In some cases the pendulum seems to have swung too far towards the provision of anaerobic conditions and perhaps to an undue emphasis on the so-called exquisite oxygen sensitivity of anaerobes; this might have been at the expense of a proper search for improved media that would satisfy the full nutritional requirements of the diverse range of anaerobes of clinical interest.

Many of the anaerobic techniques in use today utilise a combination of two or more approaches to the removal of oxygen and the production of low Eh levels in culture media. In the Hungate (1950) roll tube system and its later

modifications (Hungate, 1966; Moore, 1966), all media are pre-reduced by boiling and gassing with an oxygen-free inert gas to drive off dissolved oxygen, and a reducing agent is incorporated in the medium to ensure rapid reduction to very low Eh levels before autoclaving. Subsequent handling procedures are carried out under a stream of oxygen-free gas to maintain the reduced conditions. Pre-reduced anaerobically sterilised (PRAS) media have also been used in conjunction with anaerobic cabinet systems (Drasar, 1967). Some authors incorporate the use of gassed out syringes and roll tubes during the collection of specimens (see Holdeman and Moore, 1972; Sutter et al., 1972), in conjunction with these anaerobic culture techniques; this illustrates particular concern for the short-term oxygen sensitivity of these organisms.

The roll tube and anaerobic cabinet systems were considered to be a significant improvement on anaerobic jar techniques both for studies of normal flora (Spears and Freter, 1967; Drasar, 1967; Aranki et al., 1969) and for isolation of obligate anaerobes from clinical material (Moore, Cato and Holdeman, 1969; McMinn and Crawford, 1970). In these studies, the only workers to provide evidence based on a direct comparison of the glovebox techniques with a conventional anaerobic jar procedure were Spears and Freter (1967) and Aranki et al. (1969) and their

results showed that recovery rates of fastidious (sic) anaerobes from the normal caecal flora of mice and from human gingival flora were improved by the use of an anaerobic cabinet. Gordon, Stutman and Loesche (1971) also reported that recovery rates from gingival sulci were improved by the use of a roll tube system.

The views of Moore et al. (1969) and McMinn and Crawford (1970) were not sustained by later studies. In a comprehensive study, Rosenblatt, Fallon and Finegold (1973) obtained comparable recoveries from clinical specimens handled by anaerobic cabinet, roll tube and anaerobic jar methods. Both the Gaspak and the evacuation-replacement approaches were used to set up the jars. The same media were used throughout the study; media used in the cabinet and roll tube systems were pre-reduced and anaerobically sterilised. Similar findings were reported by Dowell (1972), Killgore et al. (1973) and by Watt, Collee and Brown (1974). Watt and his colleagues did not use PRAS media in their cabinet but freshly poured plates were reduced in anaerobic jars for 24 h before introduction into the cabinet. They showed that obligate anaerobes could be quantitatively recovered in the same numbers from human faeces using either cabinet or a standardised evacuation-replacement jar technique. Earlier studies by Collee et al. (1972) had shown that the Gaspak system was comparable to the standardised

evacuation-replacement jar approach and would grow strict anaerobes (Loesche, 1969) such as Clostridium oedematiens type D. Other studies by Collee et al. (1971) and Watt (1972) had shown that properly controlled anaerobic jar procedures were satisfactory for the culture of clinically significant anaerobes. All of the modern anaerobic culture methods have particular advantages and disadvantages. Some workers have criticised roll tube systems because of the problems encountered in inoculating roll tubes and picking single colonies from them. Anaerobic cabinet methods are said to be technically cumbersome by some workers whilst others have stated that the manipulative skills are easy to master. In this author's experience, the manipulative skills were not difficult to learn (Deacon, 1973; Deacon and Loutit, 1975). Aranki and his colleagues (1969) stated that roll tube systems had only two major advantages over open bench techniques: (i) bacteria may be transferred and cultured without significant exposure to atmospheric oxygen, and (ii) the media used may be kept at a low redox potential at all times, even at the moment of inoculation. They showed that the same criteria apply to anaerobic cabinet methods. By contrast, the anaerobic jar procedures are recognised as easy to perform and relatively cheap but they suffer from the possible disadvantage that media, specimens and isolated organisms are exposed to atmospheric oxygen

for varying period of time. Further evidence to support the views of Watt *et al.* (1974) that properly performed bench procedures are adequate for the isolation and subculture of clinically important strains of obligate anaerobes came from the studies of Loesche (1969) and Tally *et al.* (1975). As described in the *Introduction* to this thesis Loesche divided the obligate anaerobes into two major groups based on their sensitivity to oxygen. He defined as strict anaerobes species that were unable to grow on agar at pO_2 levels above 0.5% and included in this group *C. oedematiens* type D and species from the genera *Treponema*, *Selenomonas*, *Butyrivibrio*, *Succinivibrio* and *Lachnospira*. Moderate anaerobes were defined as species able to grow in oxygen concentrations from 2-8% and Loesche noted that the strains he tested could be exposed to air for periods of 60-90 min. without loss of viability. Species considered in this group were *Bacteroides fragilis*, *B. melaninogenicus*, *B. oralis* and *F. nucleatum*. Tally *et al.* (1975) distinguished between an organism's sensitivity to oxygen as defined by Loesche and its ability to tolerate exposure to air without loss of viability. These workers studied the oxygen sensitivity and tolerance of fresh clinical isolates and found that all of the isolates were able to survive exposure to air for 8 h or longer. They included individual strains of *B. fragilis*, *B. melaninogenicus*, *B. oralis* and other bacteroides strains in the category of

strict anaerobes but agreed with the previous findings of Rosenblatt et al. (1973) that clinical isolates could withstand transient exposure to air during bench handling procedures. Extremely oxygen-sensitive (EOS) strains of B. thetaiotaomicron and other B. fragilis-like strains that were unable to survive exposure to oxygen at atmospheric pressure have been described by colleagues of Tally et al. (Attebery, Nastro and Finegold, 1971; Attebery, Sutter and Finegold, 1972; 1974). Attebery et al. (1974) reported that EOS strains were present in the normal faecal flora of seven Japanese-Americans in counts ranging from 7.02×10^{8} to 1.6×10^{11}. However, Rosenblatt et al. (1973) and Tally et al. (1975) stated that they failed to isolate these strains from clinical specimens and they concluded that these strains were not clinically important.

Any consideration of the anaerobic conditions in a bacterial culture must take account of the chemical constituents in the medium that tend to be thought of mainly in a nutritional sense. The chemical constituents fulfil a potential nutrient role and they also affect the oxidation-reduction potential (Eh) developed in a culture. The Eh of a culture medium is the resultant of the interactions between all the oxidised and reduced components present. It is a measure of the net ability of the medium to donate or receive electrons and although a steady state can be achieved a true equilibrium

cannot be attained (Morris, 1975). The major determinant of Eh in a medium or culture is oxygen and in an aerated medium the Eh is directly proportional to the logarithm of the partial pressure of oxygen, or pO_2 (Squires and Hosler, 1958; Jacob, 1970).

The practical significance of these observations lies in the dependance of obligate anaerobes on defined levels of Eh for the initiation of growth from small inocula. Vennesland and Hanke (1940) showed that strains of B. vulgatus would only commence growth in a glucose broth at pH 6.6 if the Eh was less than + 150 mV and Hanke and Katz (1943) extended the concept to show that the Eh value above which growth would not occur varied for different organisms and was apparently independent of the oxygen tension of the medium. Some workers have disputed this conclusion and suggested that Eh does not have a primary role in anaerobiosis (Dack and Burrows, 1935; Futter and Richardson, 1971; Hentges and Maier, 1972). Smith (1975) concluded that all anaerobes probably have a limiting redox potential above which they cannot grow - regardless of other conditions, but noted that the determination of this value was complicated by such factors as the size and metabolic state of the inoculum and strain to strain variation within a species. Smith suggested that strict attention to this aspect of anaerobic culture was probably

most critical during primary isolation when only a few viable cells may be present in a specimen. Under such circumstances the cells will be widely dispersed and unable to reduce their immediate surroundings by the release of metabolic products or reduced metabolites during the lag phase in the new medium (Hewitt, 1950).

The methods of measuring Eh in culture media have been discussed by Hewitt (1950) and by Jacob (1970). Electrometric measurements of Eh values in culture media are commonly performed with an inert platinum electrode coupled to a standard calomel electrode and connected to a suitable potentiometer such as a millivolt reading pH meter similar to the apparatus described in the Materials and Methods section of this thesis. Jacob (1970) discussed the difficulties of calibrating these electrodes and stressed the importance of careful preparation of the platinum electrode if reproducible results are to be obtained. Other workers have used redox indicator dyes that generally decolourise progressively as conditions become more reduced. Individual dyes are reduced at different Eh values and the standard redox potentials, or E_o' values, are expressed as the Eh value at which the dye is 50% reduced (Jacob, 1970). The dependance of Eh values on pH has been discussed by Clark (1960) who has given a detailed account of many of the theoretical aspects of oxidation-reduction potentials

in organic systems. The difficulties encountered in interpretation of results were discussed by him and also by Jacob (1970).

Experimental observations on redox potentials and handling procedures

The first experiments examined the redox potentials achieved in Robertson's cooked meat medium prepared in the department. Many clinical laboratories in Scotland use this medium for the culture of anaerobes (Dye, 1975). Variation in the methods of preparation, tubing, storage and method of handling at the time of inoculation can affect the usefulness of any culture medium used for the cultivation of obligately anaerobic bacteria. Previous studies by the Edinburgh team had shown for instance that steaming liquid culture media before inoculation was an essential step for use with anaerobic jar methods of anaerobic culture (Collee, personal communication).

In describing the preparation of Robertson's CMB medium Lepper and Martin (1929) suggested that the freshly cooked meat particles should occupy a depth of about 5 cm in the bottom of a test tube and that 1% peptone broth should be added to just cover the meat. In the modification used in the present studies the cooked meat particles are covered by 10 ml of Oxoid nutrient broth no. 2 in 5 x 5/8 in.

test tubes and the depth of the broth above the meat particles is about 10 cm. It seemed that the depth of the broth above the meat or the ratio of the meat particles to the broth could affect the ability of the reducing substances in the meat to maintain the reduced conditions in the depths of the meat layer as described by Lepper and Martin. Accordingly it was decided to measure the redox potentials at two levels in representative samples of the medium.

The Eh values recorded in the aerated broth above the meat particles and in the meat layers themselves were uniformly high and at + 370 mV were similar to the findings of Jacob (1970) who examined the Eh values of similar peptone-meat broths. Lepper and Martin (1929) reported that redox indicator dyes showed that the Eh in the pink layers of the meat was about -200 mV; these findings were not confirmed in the present study. This discrepancy may in part be due to the fact that Lepper and Martin's findings were made on meat particles removed from contact with the broth and immersed in solutions of a range of redox indicator dyes. Lepper and Martin conceded the possibility that their results might not be confirmed by electrometric readings when the meat was in contact with the broth itself. They suggested that with their method of preparation convection currents in the medium were prevented by the meat and that the rate of fixation of oxygen in the vicinity of the meat

particles is greater than the rate of inward diffusion of oxygen. The relatively large volume of oxidised broth above the meat particles in the locally prepared media clearly affected conditions in the vicinity of the meat particles. Examination of a large number of prepared broths showed that the pink meat layer was restricted to a very small portion of the meat at the bottom of the tubes. An alternative explanation that the findings of the present study resulted from the transfer of the tubed media into containers that would accept the electrode system cannot be entirely discounted, but the broths were left to equilibrate for 24 h in order to minimise any effects produced by this handling.

The substantial fall in the Eh readings after steaming for 30 min. provided quantitative evidence of the effectiveness of this step in removing dissolved oxygen. The results underline the importance of oxygen in determining the redox state of liquid culture media, even in a medium that is acknowledged to possess substantial reducing substances. The average Eh in the broth layers after steaming was + 150 mV. The observation that on standing after steaming a further reduction took place showed that steady state redox conditions had not been established by the removal of dissolved oxygen but the gradient of the change, both at a depth of 2.5 cm below the surface of the broth and in the meat layer itself, suggests that the reducing substances

present were able to exert a more significant effect on the redox state in the deeper parts of the medium reducing it even further. Other observations by the Edinburgh team, instigated by the author, utilising the redox indicator resazurin, showed that re-oxidation of the surface layers in these broths occurred within a few minutes of cooling after steaming. The oxidised layer, shown by the return of the dye's pink colour, slowly extended into the deeper parts of the broth above the meat particles. The E_o' value of resazurin is -42 mV at a pH of 6.9 (Twigg, 1945).

The relatively rapid re-oxidation of the surface layers in the broths examined might be considered grounds for returning to the practice of just covering the meat particles with broth (Lepper and Martin, 1929) rather than retaining the larger volume of broth at present in use in the Edinburgh laboratory. Practical considerations make this undesirable; Robertson's CMB has been utilised successfully for routine fermentation tests (Rutter, 1970; Duerden, 1975), other biochemical characterisations and for the preparation of freeze-dried stock cultures. In all these applications the additional broth is essential. Similarly Lepper and Martin (1929) recommended the inoculation of the medium in the depths of the meat particles. This procedure has since been adopted by many workers, but in the inoculation of a series of cultures for biochemical characterisations such a procedure is impractical. The

method adopted by Rutter (1970); Duerden (1975) and subsequent workers in the Edinburgh team has been to inoculate directly on to the surface of the steamed and cooled medium and place it under anaerobic conditions without delay. These modifications have not affected the ability to grow even relatively demanding strains of clinically important obligate anaerobes. The modified broth used in these studies (see Appendix I) was found to improve the growth of a range of nutritionally demanding obligate anaerobes (Mr R. Brown, personal communication). The medium is further evaluated in relation to other enriched liquid culture media in a later section of this Discussion.

Monitoring the atmosphere of an anaerobic cabinet

Chemical, electrometric and biological methods have been used to monitor the reduced conditions existing in operational anaerobic cabinets. Lee et al. (1968) and Rosenblatt et al. (1973) used the redox indicator dye methylene blue, available commercially in the form of impregnated filter paper strips (BBL, Gaspak). Aranki et al. (1969) incorporated indicator dyes into the media used in their cabinet. The dyes used included resazurin, indigo carmine and phenosafranin. They also used an oxygen analyser in establishing the effectiveness of their plastic cabinet system but did not consider it necessary to use this device routinely. They did not state the limits of

accuracy of their analyser although the oxygen concentration was stated to be below 10 ppm during normal operation. An aerated broth contains about 9 ppm of oxygen (Jacob, 1970). Leach et al. (1971) monitored the establishment and maintainence of anaerobic conditions in their cabinet with an indicator medium containing resazurin. The open bottle of medium remained in the cabinet throughout all procedures. The authors did not comment on the effect, if any, of the 100% CO_2 atmosphere on the pH of the medium. As the E_o' value at which a redox indicator dye is 50% reduced changes with pH, this seems to be an important point. These workers also used an oxygen electrode to check the satisfactory operation of their cabinet in a single experiment. Watt et al. (1974) demonstrated electrometrically that the concentration of oxygen was below 1% in their cabinet and as a more sensitive instrument was not available these workers resorted to the use of biological indicators to check the satisfactory establishment of anaerobic conditions. Blood agar plates were seeded in the cabinet with overnight CMB cultures of Clostridium welchii, C. tetani type VI and C. oedematiens type D. C. welchii grew well at room temperature when incubated overnight in the cabinet; the other strains were transferred into an anaerobic jar that did not contain any palladium catalyst sachets and incubated at 37°C in the cabinet atmosphere. The atmosphere in the cabinet was demonstrated

to be sufficiently anaerobic to support the growth of the demanding anaerobic strains. In more recent work the Edinburgh workers routinely seeded a nutrient agar plate with a culture of Pseudomonas aeruginosa (Mr R. Brown, personal communication). The absence of any growth after leaving the inoculated plate in the cabinet at room temperature overnight was taken to indicate the establishment and maintenance of satisfactory anaerobic conditions. A similarly inoculated plate was routinely included in each anaerobic jar containing cultures for incubation at 37°C. This control is now also used in anaerobic jars set up by the standard bench procedures routinely used in the laboratory. Watt, Collee and Brown (1976) have reviewed methods of assessing the performance of anaerobic jars and recommended the use of a rapid "secondary vacuum" test for catalytic activity in conjunction with a biological indicator as described above.

Some workers do not appear to have used any method of checking that properly reduced conditions have been established and maintained during normal operation of their anaerobic cabinets. Clearly, any lack of certainty about the quality of the cabinet atmosphere prejudices any possible value that the use of such equipment might have for the handling of anaerobic microorganisms. A

common cause of problems are small undetected leaks in the gloves. Other causes of leaks that can result in significant contamination by air are loose gas and electrical lead fittings, inadequate sealing around gloves and improper closure of any airlocks or ports used to transfer materials into and out of the cabinet. Similarly the introduction of materials containing air may occur if defined handling procedures are not adhered to. Failure of the oxygen-scavenging devices used to render the flushing gas mixture free of oxygen can occur particularly if a heated tube containing copper turnings is used for this purpose and if the flushing gas does not routinely contain hydrogen to maintain the reduced state of the absorbent material. The 100% carbon dioxide used by Leach et al. (1971) is an example of a situation where this could occur. Some workers rely on trays of palladium coated pellets within the chamber to remove any contaminating traces of oxygen that might gain access, but if these are not changed or rejuvenated regularly they too can fail.

In the course of his M.Sc. studies the present author used an anaerobic cabinet based on the design of Leach et al. (1971). He saw the need for a continuous monitoring system capable of detecting even minor degrees of contamination to the atmosphere in a cabinet (Deacon, 1973).

Oxygen analysers capable of detecting very low levels of oxygen in a mixture of gases are expensive and as the

planned studies in Edinburgh involving a cabinet were of a limited nature such a device could not be considered. In addition, the findings of Rosenblatt et al. (1973) and Watt et al. (1974) that anaerobic cabinets could not be shown to be of practical value to the clinical bacteriologist seemed to indicate that it was unlikely that the expense of oxygen analysers could be justified by their possible extensive use in the future.

A continuous monitoring device can help to shorten the period of time taken to establish the properly reduced conditions necessary for normal operation. It provides an immediate indication if the ideal working conditions are compromised; this is a positive reassurance when all is well and obviates the need for incorporation of possibly toxic dyes in culture media. Nevertheless, it seemed that an inexpensive alternative could be developed by adaptation of the electrometric device used for determining Eh values in culture media. Clark (1960) had noted that a platinum electrode near the surface of a culture may behave deceptively like an oxygen electrode and he stated that empirical use of this had been attempted in the past. Further evidence to support the view that such an approach was possible came from a number of workers. Wimpenny (1970) noted that small changes in the concentration of oxygen in bacterial cultures could be registered by an Eh electrode

whereas a sensitive Mackereth-type oxygen electrode barely responded. The dependance of redox potentials in culture media on the partial pressure of oxygen noted by Squires and Hosler (1958) and by Jacob (1970) was referred to earlier. The findings of Schuldiner, Piersma and Warner (1966) are also important in this respect; these workers demonstrated that in a gas-tight system with negligible oxygen leak the potential on a platinum electrode was proportional to the pO_2 up to pressures of 10^{-9} atmospheres.

It is clear that caution is required in the interpretation of results obtained with Eh measuring devices; Clark (1960) has discussed many aspects of this very complex field and Jacob (1970) has made many thoughtful comments and given very useful technical advice. The device developed in these studies was relatively simple and inexpensive to set up and maintain. The preliminary trials reported indicate that response to contamination by air is rapid and that the system is also sensitive to a reduction in the gas flow below a critical level. It was not affected however by changes in pressure induced by manipulation of the gloves. Further studies are required to determine more precisely the rate and magnitude of the response to defined amounts of oxygen.

The Edinburgh team concluded in the early 1970's that there was no evidence that the use of an anaerobic cabinet would be of value to clinical bacteriologists but the proviso was added that if this view was challenged there is an obligation to define clinically important groups of organisms likely to occur in clinical specimens that could only be isolated by specialised procedures (Watt, 1972; Watt et al., 1974). To date at July 1977 no one has challenged this view.

The present author considered that there was a need to extend the earlier observations by testing additional strains to determine whether the growth of clinically important strains in liquid media could be quantitatively affected by differences in the anaerobic culture methods. The results of the limited studies undertaken showed that the strains tested were not affected by such differences. The type strain of B. ruminicola was included because strains belonging to this species have been frequently isolated from human infections (Smith, 1975). Although no pathogenic role has been identified for strains of G. formicilis these organisms were only recently isolated and characterised from human faeces (Gossling and Moore, 1975) and little is known of their frequency of distribution or their ecological significance.

It was recognised that all the strains tested were

from reference culture collections and that they may have adapted to laboratory culture but there is little in the literature to indicate that major changes occur in the oxygen sensitivity or tolerance of strict anaerobes. Loesche (1969) used reference cultures of C. oedematiens type D when he described this subgroup as strict anaerobes incapable of growth in oxygen concentrations above 0.5%. Tally et al. (1975) attempted to test the oxygen tolerance and oxygen sensitivity of fresh clinical isolates with a minimum of subcultures. They concluded that further studies were required to determine whether or not obligate anaerobes become more oxygen tolerant on repeated subculture.

The nutritional requirements of the family Bacteroidaceae

The nutritional requirements of the Bacteroidaceae are known to be varied. Varel and Bryant (1974) showed that strains of B. fragilis and the closely related species of B. vulgatus, B. thetaiotaomicron, B. distasonis and B. ovatus have substantial biosynthetic abilities being capable of growth on a simple defined medium containing glucose, haemin, vitamin B_{12}, minerals, bicarbonate-carbon dioxide buffer, ammonium chloride and sulphide. They found that more complex nitrogen sources including single amino

acids, nitrate, urea, peptides or mixtures of L-amino acids were not as efficiently utilised as ammonia. Cysteine was an excellent source of sulphur but thioglycollate was a poor source, and other sulphur compounds tested were not utilised at all. The vitamin B_{12} requirement was replaceable with the amino acid methionine at a concentration of 7.5 µg/ml.

Caldwell and Arcand (1974) have shown that ruminal and non-ruminal Bacteroides spp. have specific requirements for tetrapyrrole compounds such as haem, for iron (Fe^{2+}) and for other metallic ions including Mg^{2+}, Ca^{2+}, Co^{2+} and Mn^{2+}. Sodium (Na^{+}) ions were an absolute requirement of rumen strains; the Na^{+} requirement of non-ruminal strains could be partially replaced by Li^{+} or Cs^{+}.

Some ruminal and human oral isolates of B. melaninogenicus have been shown to require vitamin K or related compounds such as menadione (vitamin K_3) and also haemin (Lev, 1958 and 1959; Gibbons and MacDonald, 1960; Lev et al., 1971). Lev et al. (1971) found that sodium succinate could replace the haemin requirement of their rumen strains and that it could also partially replace the vitamin K requirement when added to media along with haemin. The addition of all three growth supplements together increased the growth rate of their strains. They suggested a central role for

succinate in the metabolism of the strains and demonstrated that the growth-stimulating effect was specific for succinate. Cells grown in the presence of haemin, vitamin K and succinate were not sensitive to cyanide.

In other respects also, strains of B. melaninogenicus appear to have more complex growth requirements than strains of B. fragilis. Preliminary studies by Varel and Byrant (1974) showed that strains from each of the three major subspecies of B. melaninogenicus were unable to grow in the minimal defined medium developed in their studies on B. fragilis. The heterogeneous nature of the group creates some difficulties when attempting to interpret the earlier literature. Sawyer et al. (1962) recognised at least two nutritional types, a saccharolytic group of strains that fermented carbohydrates and a proteolytic but non-saccharolytic group of strains that grew well in media without carbohydrate supplementation. Wahren and Gibbons (1970) later demonstrated that asaccharolytic strains fermented amino acids and that addition of glucose did not stimulate growth although it was utilised poorly. Their strains possessed only limited ability to ferment free amino acids but grew well in a medium containing the peptide-rich nutrient Trypticase (BBL). Amino acid fermentation has also been reported for strains of Fusobacterium nucleatum (Jackins and Barker, 1951; Loesche

and Gibbons, 1968). With both the B. melaninogenicus strains and the F. nucleatum strains, catabolite repression of amino acid utilisation by glucose intermediates does not occur (Wahren and Gibbons, 1970).

An apparent requirement for peptides has also been reported for strains of B. ruminicola (Pittman and Bryant, 1964; Pittman, Lakshmanan and Bryant, 1967). All the strains tested by Pittman and Bryant (1964) grew well in a defined medium containing glucose, minerals, B vitamins, haemin, volatile fatty acids, methionine and cysteine with ammonia as the main source of nitrogen. The strains were able to utilise peptide nitrogen in the form of Trypticase but were unable to utilise significant amounts of free amino acid nitrogen.

Growth studies with B. melaninogenicus ss. asaccharolyticus

The growth studies were undertaken because an increasing number of growth failures were occurring with the media used for identifying and characterising reference and fresh clinical isolates of the species B. melaninogenicus. In particular the thioglycollate medium previously adopted for routine carbohydrate fermentation tests by Duerden et al. (1976) was proving to be inadequate. In the earlier studies the medium had performed well in the

characterisation of mainly reference and clinical isolates of B. fragilis and similar non-pigmented Bacteroides species, but as more demanding strains were isolated the number of failures increased (Mr R. Brown, personal communication). At this time also Dye (1975) reported that he found Brewer's thioglycollate medium (Oxoid) inferior to a supplemented Brain Heart Infusion broth and two commercially prepared cooked meat media for the culture of clinical isolates of a range of obligately anaerobic species. Similar findings were reported by Rosenblatt et al. (1973) who compared two other commercially prepared thioglycollate media with PRAS cooked meat broth also commercially prepared. The growth studies were also planned to develop new media required for other purposes including GLC studies of the fatty acid metabolic products of members of the Bacteroidaceae.

The choice of B. melaninogenicus ss. asaccharolyticus as a model for the planned growth studies was suggested by the finding that some strains from this subspecies were particularly difficult to grow in some of the media in use at the time. The decision was also influenced by recent suggestions that the most common isolates of B. melaninogenicus from clinical specimens were of this subspecies (Holdeman et al., 1974) and by the knowledge that, because these strains are proteolytic and do not

require added carbohydrate, the effects of various peptones and growth factors could be evaluated more simply. By restricting testing to a small group of similar strains, providing a spectrum ranging from the relatively easy to grow to the very fastidious and difficult to grow, it was possible to simplify the design of the experiments yet still obtain meaningful results, the conclusions of which might be applied to a wider range of strains from the same family.

The measurement of bacterial growth in culture media may be accomplished in a variety of ways. Sophisticated techniques for determining bacterial mass such as estimations of DNA, RNA, nitrogen and protein content are time-consuming and technically difficult in comparison to total or viable cell counts and visual or spectrophotometric measurements of turbidity. Meynell and Meynell (1970) and others have described the various approaches and outlined the possible sources of error associated with the different methods. Semi-quantitative estimations of visual turbidity and spectrophotometric measurements of turbidity were considered adequate for the purposes of the present studies. These approaches were correlated with total cell counts in one experiment and the conclusions of this experiment will be discussed below.

The simple visual assessments were found to be particularly useful in dealing with a large number of cultures. They were most effective in discriminating between cultures where growth was poor or barely adequate and cultures where good growth (3+ levels or above) occurred. Discrimination between cultures showing high levels of turbidity was less reliable. It was realised that such assessments are subjective and the preparation of a standard set of tubes showing six selected levels of turbidity was designed to minimise any variations in interpretation. The six grades selected represented significant observable differences in the growth levels of the different test strains. Where more precise discrimination was required in some experiments, spectrophotometric readings were performed.

It was not considered that Brown's opacity tubes would offer any particular advantages. These tubes, containing graded amounts of barium sulphate, are commonly used to determine the approximate number of cells in a prepared suspension and they require calibration for each strain tested (Cruickshank et al., 1975). They are also subject to the same sorts of error that can affect the accuracy of simple turbidity measurements (see Cruickshank et al., 1975) and have the additional problem that if used

for assessing approximate cell numbers by direct comparison with bacteria suspended in the growth medium there is no compensation possible for the colour of the medium or for the presence of crystals or particulate debris in it. In the visual and spectrophotometric readings performed in the present studies the uninoculated medium was considered to be an essential control.

It is generally known that growing bacteria produce a turbidity in liquid media because light is scattered by the suspended bacteria and the amount of light scattered at the interface of cells and medium is proportional to the concentration of cells, expressed either as mass or number, and is affected by the size and shape of the cells and the conditions of growth (Meynell and Meynell, 1970; Cruickshank et al., 1975). The loss of linearity observed in the absorbance readings with increasing cell numbers is caused by secondary scattering of the light and the Lambert-Beer Law no longer applies (Meynell and Meynell, 1970).

The experiment correlating total cell counts and culture turbidity with four bacteroides strains confirmed the observations of many workers that significant strain to strain variation occurs in the amount of turbidity produced in a given medium grown under the same conditions

for the same period of time. The most significant difference was seen at genus level; after incubation for 48 h the three strains from the genus Bacteroides produced higher levels of growth, as shown by total cell counts, and produced more turbidity for a given number of cells than the single representative of the genus Fusobacterium. There were also differences between the three Bacteroides strains with the most turbidity produced by the strain of B. fragilis. The B. melaninogenicus ss. asaccharolyticus strain produced more turbidity than the strain of B. melaninogenicus ss. intermedius. Detailed results showed that turbidity changes were registered over a relatively small range of total cell counts. There was a rapid loss of linearity in spectrophotometric response with increasing cell numbers particularly at absorbance values above about 1.00 (see fig. 10).

The apparent unreliability of the spectrophotometric readings noted at absorbances of 0.020 or less (see table 11, Results section) suggests that this value represents the lower confidence limit of this approach and that under these conditions of analysis an initial absorbance change of > 0.020 must occur in an inoculated medium before it could be reliably concluded that growth, or more precisely cell division, had taken place. This value was clearly not the same as the minimum difference that could be detected

between cultures once growth had occurred. The reproducibility of readings on a single culture with the Pye-Unicam SP600 spectrophotometer was considered to be ± 0.005 on readings below 0.25 and ± 0.01 on readings between 0.25 and 1.0.

The PY medium first described by Moore et al. (1966) has become widely accepted as a general purpose basal medium useful in routine identification testing of obligate anaerobes. The medium has been used by research and clinical workers to test the phenotypic reactions of their isolates to a wide range of added carbohydrates and other possible growth factors; it has also been used for the gas chromatographic analysis of fatty acid metabolic products of fermentation as described by Holdeman and Moore (1972).

The present studies showed that the medium was unsatisfactory for the growth of some strains of B. melaninogenicus ss. asaccharolyticus and these results confirmed earlier observations made when the writer attempted to use the medium for gas chromatographic analyses of the fatty acid metabolic products of a range of clinical isolates from the genus Bacteroides. The growth studies showed that a wide range of growth supplements were unable to stimulate growth of the test strains in PY medium, but good growth was obtained in more enriched media that did

not contain the balanced salts solution of Holdeman and Moore (1972).

The balanced salts solution inhibited the growth of representative strains of B. melaninogenicus ss. asaccharolyticus when it was added to ½PPY medium. The growth of glucose-fermenting strains of B. melaninogenicus was not affected. Further studies are required to elucidate the cause of the inhibition observed. This aspect was not pursued in the present studies as it was considered that such an investigation could prove time-consuming and it was clearly not essential to the growth of the strains tested or to the development of a range of improved media suitable for characterisation studies.

There were substantial strain to strain differences in the levels of growth achieved by strains of B. melaninogenicus ss. asaccharolyticus in ½PPY or ½PPYG media. The type strain NCTC 9337 produced no more than adequate levels of growth, and fresh clinical isolates including the Bangour test strains 2296 and 3502 grew poorly on prolonged incubation; despite this, the media were seen to be a significant improvement on PY or PYG media. In PPY medium without glucose, the NCTC strain did not reach high population levels even after incubation for seven days and first signs of growth were not apparent until the second day of incubation. The addition of glucose to the medium

($\frac{1}{2}$PPYG medium) caused an additional delay of about 24 h before the appearance of observable growth. A similar observation of a glucose-induced lag has been reported in asaccharolytic rumen strains of B. melaninogenicus by Lev and Milford (1975) and they concluded that with their strains sensitivity to monosaccharides resulted from an inhibitory effect of low concentrations of sugars such as glucose, galactose, mannose and fructose on the induction of one or more key enzymes. For instance, they found that addition of glucose or galactose abolished the induction of 3-ketodihydrosphingosine synthetase by vitamin K in vitamin K-depleted cells and that levels of the enzyme were much reduced in cells not depleted of vitamin K. The mechanism of inhibition was not elucidated. In the present studies it was found that glucose caused a prolonged lag phase, but once growth was under way the growth rate and the population levels achieved in $\frac{1}{2}$PPYG medium were similar to those in $\frac{1}{2}$PPY medium. Lev and Milford (1975) found that the monosaccharide-induced growth inhibition of their strains was overcome on prolonged incubation for 2-3 days.

The growth studies and later experience in vitamin B_{12} growth studies, GLC, and fermentation studies provided clear evidence that Proteose peptones were significantly better nutrients for a wide range of saccharolytic and

asaccharolytic strains from the family Bacteroidaceae than the ordinary bacteriological peptones used by some previous workers. Both carbohydrate fermenting and non-fermenting strains grew in ½PPY and ½PPYG media and an increase in the concentration of Proteose peptone to 2%, as in PPY and PPYG media, further improved the growth of a wide range of clinically important strains and often yielded very high populations of cells from small starting inocula. The addition of horse serum to these media improved the growth of many strains even further.

It is not possible to determine the precise reason or reasons for the growth differences noted for any particular strain in different complex culture media but it is generally accepted that the better nutritional value of Proteose peptones in comparison with ordinary peptones results from milder methods of preparation. Bacteriological peptones are produced by enzymic hydrolysis of mammalian muscle tissue and, as the Proteose peptones are less hydrolysed than ordinary peptones, labile growth factors are conserved along with a higher proportion of small or medium-sized peptides that may be more easily assimilated by some bacterial strains (Meynell and Meynell, 1970).

In general, serum improved the growth of many of the strains examined. The addition of serum to complex culture

media has been recommended by some workers and avoided by others. Meynell and Meynell (1970) have reviewed the findings of various workers showing that it may (i) act non-specifically as an absorbent for fatty acids and other possibly toxic materials and in this role it can be replaced by charcoal; (ii) act as a buffer; (iii) contribute unspecified quantities of growth supplements and nutrients such as fatty acids, vitamins, minerals, amino acids or other nitrogenous compounds, lipids and small amounts of carbohydrates; (iv) supply enzymes such as maltase and invertase. Clearly in a general purpose enrichment medium it may be beneficial but it cannot be used in media required for specific tests, the results of which may be affected by its addition. In the present studies the effect of its addition or deletion was determined in various applications.

The present studies confirmed the effectiveness of Trypticase as a nutrient able to stimulate the rapid growth of a wide range of nutritionally demanding strains of B. melaninogenicus and related species to high population levels particularly when used in combination with another proteose peptone and a yeast extract. The complete BM medium used in the present studies differed from the original medium described by Williams et al. (1975) in the deletion of glucose and the substitution of horse serum for bovine serum. The concentrations of the major

constituents remained the same. It proved to be an excellent culture medium for a wide range of fresh clinical isolates and reference or type culture collection strains. The combination of Trypticase, Proteose peptone (Oxoid) and Yeast extract (Difco) was shown to be particularly useful but the substitution of Tryptone (Difco), another peptide-rich tryptic digest, for Trypticase, caused only minor decreases in the growth of the strains tested. The substitution of one brand of Proteose peptone (Difco, no. 3) for another (Oxoid) made no difference. Single deletions of any of the three major nutrients significantly affected the growth of the test strains. Increasing concentrations of Trypticase or Yeast extract in BM medium from which one or the other had been deleted produced corresponding increases in the growth although the differences between 1% and 2% were minimal in both cases. The addition of fresh meat particles increased the growth rates of virtually all the strains tested and the single deletion of horse serum caused an increase in the lag phase of the same strains. Added glucose improved the growth of glucose-fermenting strains. Casamino acids could not substitute for Trypticase.

In general, despite strain to strain differences, complete BM medium supported more rapid growth to high population levels than any other version or similar medium

except that of BM itself enriched by meat particles. Demanding strains of B. melaninogenicus ss. asaccharolyticus generally grew best in complete BM medium containing meat particles or in Robertson's CMB enriched with Trypticase.

The detailed comparisons performed in arriving at the above conclusions were considered an important part of the evaluation of new media intended for use in extensive characterisations of a nutritionally demanding and heterogeneous group such as the Bacteroidaceae, because minor changes in the composition of a complex culture medium can have significant effects on the ability of the medium to grow fastidious strains and various types of digest can differ markedly, especially when used in liquid form (Meynell and Meynell, 1970).

The composition of BM medium is similar to that of Schaedler broth (Schaedler, Dubos and Costello, 1965) but the concentrations of the major nutrients are higher. Stalons, Thornsberry and Dowell (1974) compared Schaedler broth and eight other commercially available media, including two defined media, in tests with four representative strains of anaerobic bacteria from human infections. Broths, gas mixtures and inocula were evaluated for use in developing a procedure for performing minimal inhibitory concentration antimicrobic susceptibility tests. The results demonstrated

the clear superiority of Schaedler broth in an atmosphere of 5% carbon dioxide, 10% hydrogen and 85% nitrogen. Growth curves plotted for the four strains showed that high growth rates under these conditions yielded dense populations after incubation for 24 h. An increase in carbon dioxide concentration to 10% was helpful for only one strain, an anaerobic coccus. A possible criticism of this study is that the range of strains tested was not particularly demanding, but additional growth curves obtained with more demanding strains appeared to confirm the original findings. Stalons et al. noted that more fastidious strains such as the anaerobic cocci might require further supplementation but their findings with a single non-fermenting strain may have been adversely affected by the lower concentrations of Trypticase and other major nutrients in the Schaedler broth.

Further evidence underlining the value of BM or similar media for growth of demanding strains of B. melaninogenicus came from studies complementary to the present work. Holbrook (1976) tested a further modification to BM broth for the recovery of clinical isolates from dental plaque samples and compared his modification with other enriched broth media. The media tested included: (i) modified Robertson's CMB; (ii) thioglycollate medium without dextrose

or indicator (BBL) but containing haemin, menadione, sodium succinate and yeast extract; (iii) brain-heart infusion broth (Oxoid) with haemin and menadione. (iv) BM broth (Williams et al., 1975); (v) modified BM broth containing sodium succinate; (vi) a modified medium E (Holdeman and Moore, 1972; as modified at the present author's suggestion); (vii) horse digest broth with haemin and menadione, and (viii) NCTC tissue culture medium, NCTC 109 TC containing haemin and menadione. A range of demanding strains of B. melaninogenicus, some of which were also used in the present studies, grew best in the modified BM medium and in the modified medium E. As the preparation of the BM medium was simpler, Holbrook (1976) used this medium for clinical sampling.

Studies with vitamin B_{12}

Vitamin B_{12} is an essential growth factor of all living organisms. It is readily synthesised by many microorganisms including bacteria or other microorganisms growing in the soil or water, or in the rumen or intestine of animals. Since its isolation in 1948 it has been the subject of intense research and its complex chemical structure has been established by X-ray crystallography and chemical degradations. In his monograph, Lester Smith (1965) gives a detailed and balanced account of all aspects of the vitamin and its coenzymes.

The vitamin B_{12} molecule (cyanocobalamin) consists of a porphyrin-like planar group and a nucleotide that lies almost at right angles to the planar group. The base of the nucleotide is 5,6 dimethylbenziminazole; the sugar is ribose and it is phosphorylated at C3. The planar group has a central cobalt atom linked to four reduced pyrrol rings. The vitamin B_{12} coenzyme contains the whole cobalamin molecule linked to adenosine by a cobalt-carbon organo-metallo bond (Lester Smith, 1965). The coenzyme form is extremely sensitive to light but cobalamin itself is relatively stable. A number of naturally occurring and synthetic analogues are now known and these were listed by Lester Smith. Some analogues are biosynthesised by feeding fermenting bacteria with the appropriate substitute bases.

Several vitamins occur naturally bound to peptides or proteins and it has been established that vitamin B_{12} is readily bound in vivo to a labile enzyme-like substance the 'intrinsic factor' of Castle and secreted by the normal stomach of Man and some animals (Castle, 1929 cited by Lester Smith, 1965). The binding of vitamin B_{12} to Castle's intrinsic factor assists its absorption from the gut. A deficiency of vitamin B_{12} or folic acid in the diet or a failure to absorb the vitamin due to non-

production of intrinsic factor can cause megaloblastic anaemias. There is also an absorptive failure associated with blindloop syndrome and tropical sprue (Drasar and Hill, 1974).

During the growth studies in the present work it was found that the growth of three strains of B. melaninogenicus ss. asaccharolyticus was stimulated by the addition of a high concentration of vitamin B_{12} to cultures in various Proteose peptone enriched media. The test strains of B. melaninogenicus ss. asaccharolyticus included the type strain NCTC 9337 and two recent clinical isolates, Bangour strains 2296 and 3502. In early studies the final concentration of the vitamin in the $\frac{1}{2}$PPYS test medium was 1×10^7 pg/ml. Initial observations with test tube cultures inoculated on the bench and incubated in anaerobic jars were quantitatively confirmed with spectrophotometric turbidity readings taken on samples withdrawn after gentle mixing of unstirred bulk cultures held under extremely reduced conditions. The inocula for the bulk cultures were calculated to ensure that the ratio of inoculated cells to medium volume was similar to that used with the test tube cultures. There were differences in the time taken to obtain good levels of growth by the different approaches, but in both cases the addition of the vitamin clearly stimulated better growth to higher population levels. It was found that the serum in $\frac{1}{2}$PPYS medium produced an additive effect on the

growth stimulation caused by addition of the vitamin and accordingly this serum supplement was excluded from all further investigations.

Other species or subspecies from the genus Bacteroides were not stimulated by the addition of the vitamin to the test media. The strain designated B. oralis, ATCC 15930 (B. melaninogenicus) was inhibited by the vitamin at a concentration of 1×10^7 pg/ml in $\frac{1}{2}$PPY medium. An examination of the effects of different concentrations of the vitamin on the growth of six B. melaninogenicus strains including this strain and the two clinical isolates of B. melaninogenicus ss. asaccharolyticus showed that the B. oralis strain was inhibited by the vitamin at concentrations of 1×10^5 pg/ml or above; the test strains of B. melaninogenicus ss. asaccharolyticus were maximally stimulated at concentrations of 1×10^4 pg/ml or above and three representative strains from other subspecies of B. melaninogenicus were not affected by addition of the vitamin at any concentration. The lowest concentration tested was 10 pg/ml.

The growth of the B. melaninogenicus ss. asaccharolyticus strain Bangour 2296 was also tested in PPY medium and in that medium containing vitamin B_{12} at concentrations ranging from 1×10^4 pg/ml to 1×10^7 pg/ml with incubation times up to 48 h. The earlier results were confirmed;

good stimulation of growth occurred at each of the vitamin concentrations tested. The growth curves plotted in fig. 14 in the Results section showed that the lag phase in both the vitamin-free PPY cultures and in the vitamin-enriched cultures was similar. First signs of growth in all media occurred after incubation for 24-30 h but maximum rates of exponential growth in the vitamin-enriched media did not become properly established until after incubation for 36-40 h. Exponential growth did not occur in the vitamin-free PPY medium during the period of the experiment. Total cell counts performed on the cultures in PPY medium and the cultures containing vitamin B_{12} at a concentration of 1×10^5 pg/ml showed that maximum cell increases occurred in the vitamin-enriched media after incubation for 40 h.

A comparison of these findings with the earlier results obtained with the same strain in the bulk culture experiment shows that in the latter the lag phase was much shorter in what were similar media to that used in the most recently described experiment. In the bulk cultures the strain of B. melaninogenicus ss. asaccharolyticus produced visible signs of growth in $\frac{1}{2}$PPYS medium, in vitamin enriched $\frac{1}{2}$PPYS medium and in BM medium after incubation for 14 h but in the anaerobic jar experiment the same strain took 24-30 h to show signs of growth in PPY and vitamin-enriched PPY medium. The inoculum was

similar in both experiments. In the bulk cultures maximum rates of growth were reached very quickly after the initiation of growth in all the media but in the anaerobic jars there was a relatively long delay from the time when growth was first observed to the time when maximum rates of growth occurred in the vitamin-enriched media only.

Differences in the media used in the two experiments may be partly responsible for the variations observed between the two studies but it is more likely that differences in the handling procedures were more important factors. The bulk cultures were inoculated when the media were already at the optimum temperature for growth and reduced to very low levels of redox potential. It is well known that the lag phase of inoculated cultures incubated in anaerobic jars is prolonged as a result of the time taken for the media to warm up to 37°C and by the time taken for adequately reduced conditions to be produced. The presteaming of the media before inoculation and the standardised anaerobic jar procedures of Collee et al. (1972) would help to minimise at least part of the delay but the introduction of oxidised supplements at the time of inoculation would tend to have the opposite effect. Conversely it is also accepted that differences in the handling procedures used for bulk cultures must have an effect of the results

obtained with this approach; whether for instance the cultures are stirred continuously, shaken or left unstirred and whether the gassing probe lies above the surface of the medium or is immersed and producing bubbles. The existence of such variables might indicate that anaerobic jars are not suitable for use in growth experiments of this type, but the results suggest that proper attention to details of procedure can yield meaningful growth curves that are comparable with results obtained from bulk cultures although the time scale is different. For practical purposes the anaerobic jar methods were preferred and it appears that the metabolism of the test strain of B. melaninogenicus ss. asaccharolyticus was not adversely affected by the prolonged exposure to slightly less than optimum conditions that may have occurred in the cultures handled in this way. This has practical significance in relation to arguments that have perhaps over-stressed the need for anaerobic cabinets in work with bacteroides organisms.

Growth curves were also prepared from duplicate cultures in anaerobic jars of the three test strains of B. melaninogenicus ss. asaccharolyticus in $\frac{1}{2}$PPY, PPY and BM-S media and in the same media containing vitamin B_{12} at a final concentration of 1×10^5 pg/ml. Cultures were incubated for a range of intervals up to 46 h.

First observable signs of growth were seen in general after incubation for 16-30 h. There were strain to strain differences in the rate of growth and the population levels reached in the different media. With the exception of the type strain NCTC 9337 in ½PPY medium, growth rates were higher in the vitamin-enriched media. The Bangour strain 2296 produced excellent growth in vitamin-enriched BM-S and PPY media and reasonable growth in BM-S medium without the vitamin, but growth in the remaining media was poor. The other Bangour strain 3502 produced good growth in the vitamin-enriched BM-S medium and fair growth in the vitamin-enriched PPY and ½PPY media, but growth in all vitamin-free media was poor and it was almost non-existent in ½PPY medium.

These differences were consistent with previous observations and illustrated the differing degrees of fastidiousness exhibited by these strains throughout the growth studies. The results with the vitamin-free media were also considered to indicate that in these otherwise enriched media the strains were either unable to obtain adequate amounts of utilisable nutrients to produce the energy necessary for biosyntheses and high rates of growth, or alternatively to obtain sufficient quantities of an essential growth factor necessary for rapid growth. The

finding that the growth of these strains could be improved by addition of high doses of vitamin B_{12} or horse serum at a final concentration of 2% in the various media demonstrated that more rapid growth is possible and that the slow growth in the unenriched media is not an inherent characteristic of the strains. The findings suggest that although these media are a clear improvement on simpler media based on ordinary peptones there is some additional factor introduced by horse serum or high doses of vitamin B_{12}.

Two radioactive labelling experiments with ^{57}Cobalt-labelled vitamin B_{12} cyanocobalamin demonstrated that the test strains of B. melaninogenicus ss. asaccharolyticus, NCTC 9337 and Bangour strains 2296 and 3502, were able to take up and bind large quantities of the vitamin from PPY medium during anaerobic incubation at 37°C. Little or no label was taken up in the first 4 h of incubation despite the use of a 37°C water bath to minimise warm-up time for these cultures. More than 70% of the labelled vitamin was taken up within a 24-h period and as the concentration of the vitamin used was 1×10^3 pg/ml this represented an average utilisation of > 700 pg/ml/24 h. The percentage recovery experiment confirmed that the loss of the label from the medium correlated with uptake

by the cells and showed that the average utilisation by the three strains over a 48-h period was 759 pg/ml. In both experiments the test strains showed clear evidence of growth in the test media after incubation for 24 h, but a comparison of test cultures and vitamin-free controls failed to demonstrate any growth stimulation at the concentration of vitamin used.

Schjonsby and Hofstad (1972) have previously demonstrated that strains of E. coli and B. fragilis are able to take up intrinsic factor-bound vitamin B_{12} in vitro and that pre-incubation of the bound vitamin with populations of these strains reduced intestinal uptake and absorption of the vitamin from the mixture in rats. They concluded that such evidence suggested that uptake by intestinal bacteria of bound vitamin B_{12} may contribute to the malabsorption of the vitamin in the blind-loop syndrome. The present studies provide evidence that intestinal strains of B. melaninogenicus ss. asaccharolyticus could also play a significant role in this condition. Schjonsby and Hofstad (1972) found that their strain of B. fragilis took up 48.9% of a 1.25 ng/ml labelled dose of rat gastric juice-bound vitamin B_{12} after incubation for only 6 h. The negligible uptake by the strains of B. melaninogenicus ss. asaccharolyticus

in 4 h in the present study suggests a widely different rate of absorption, but it is possible that binding of the vitamin to intrinsic factor would enhance its uptake by these strains also. In addition it is not known whether the free vitamin B_{12} added to the PPY medium in the present studies was subsequently bound to peptides in the medium which could also have affected the rate of uptake by the cells.

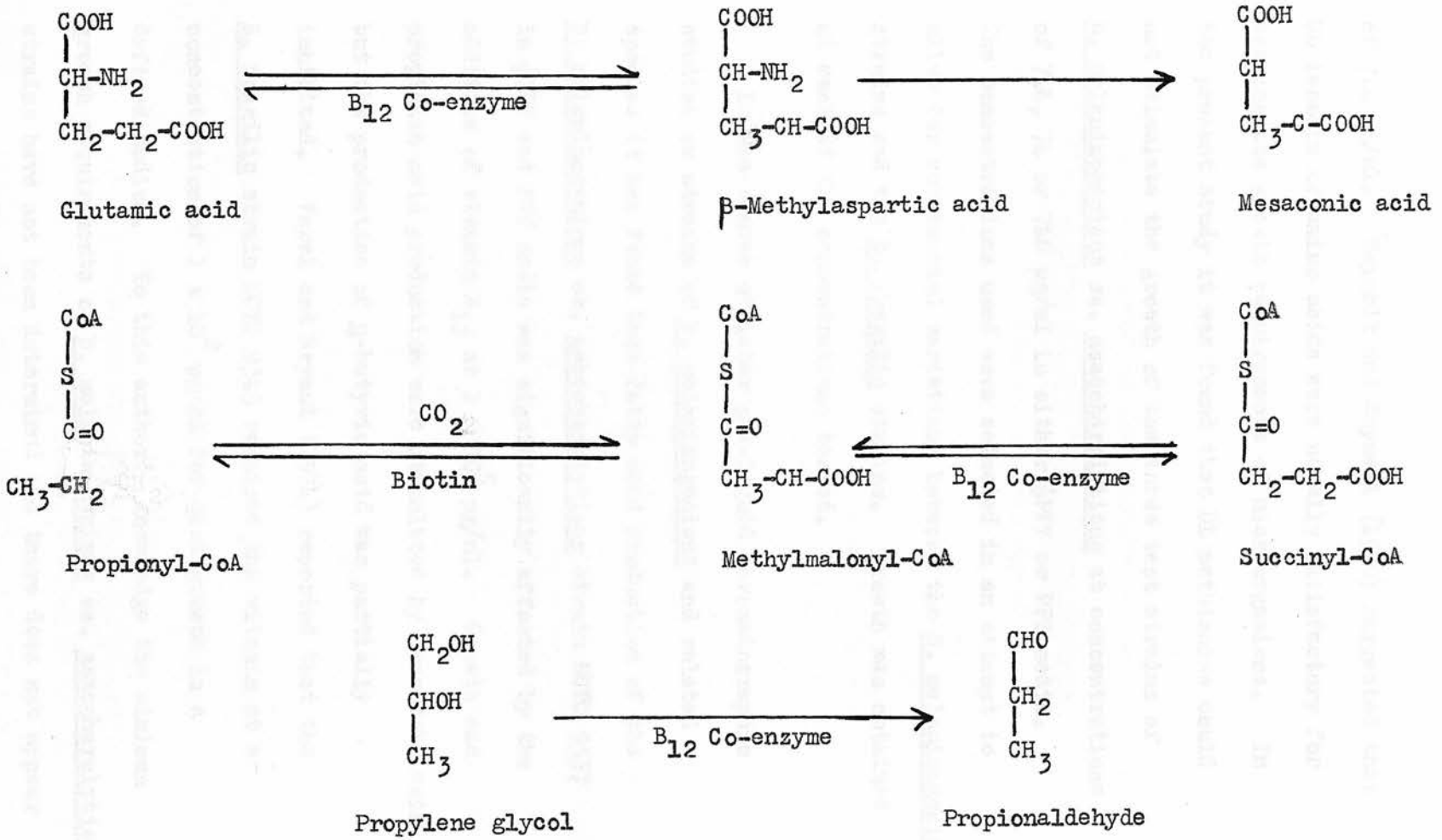
The mechanisms by which serum or high doses of vitamin B_{12} could stimulate growth were not elucidated. Further study of the stimulation by vitamin B_{12} will require the use of a defined medium because the overriding difficulty with growth studies in complex media is that absolute requirements for a particular growth factor by a given strain cannot be determined with certainty and uncontrolled and unknown effects can occur. The present author is not aware of a suitable defined medium for these strains; Varel and Bryant (1974) noted that their defined medium for B. fragilis and its related species was unsuitable for strains of B. melaninogenicus and suggested that this might have been caused by a requirement for more B vitamins or vitamin K, more complex nitrogen sources or for other unknown reasons. Other workers have also commented on the difficulties of

developing suitable defined media for bacterial strains that have an apparent requirement for peptides or other complex nitrogen sources. In the limited time available the present author was unable to develop this aspect further. Trials with a variety of defined media were instigated but were not successful. The findings were not reported in the Results section of this thesis because they were considered to need more extensive and detailed study.

Meynell and Meynell (1970) have suggested that if a growth supplement is effective only in high doses then it is likely that it is acting in a non-specific manner; they cited the well known abilities of serum to act in this way, but the radioactive labelling experiments performed in the present studies showed that the test strains of B. melaninogenicus ss. asaccharolyticus were taking up large quantities of vitamin B_{12} over a period of 24 h or less. Such findings appear to suggest that the growth stimulatory effects noted for the vitamin were not simply due to a non-specific absorption or to an alteration in the balance of the medium, as suggested to occur with the addition of some amino acids (Meynell and Meynell, 1970). It seems more likely that the growth enhancement resulted from the stimulation of one or more biosynthetic pathways.

Many diverse functions have been ascribed to vitamin B_{12} and its co-enzymes in the metabolism of bacterial cells. Lester Smith (1965) and other have reviewed the known and suggested roles that the vitamin or its co-enzymes may have and they include: (i) the protection of reduced sulphydryl compounds or the reduction of sulphydryl group-containing enzymes or co-enzymes concerned with carbohydrate or lipid metabolism; (ii) synthesis of nucleic acids including synthesis of the deoxyribose moiety and the synthesis of purines and pyrimadines; (iii) the synthesis of the amino acid methionine and possibly through it the control of protein metabolism; one attractive hypothesis directs attention to its possible role in the control of the synthesis of diverse apo-enzymes; (iv) involvement in one carbon transfers in conjunction with tetrahydrofolic acid, as for example, in glycine-serine interconversions; (v) catalysis of isomerase reactions by the co-enzyme form (see fig. 15); (vi) catalysis of certain dehydrase reactions by the co-enzymes in some species of bacteria, and (vii) other possible roles in lipid metabolism and in linked reactions with other vitamins or folic acid. Varel and Bryant (1974) showed that the amino acid L methionine could replace the vitamin B_{12} requirement of the B. fragilis strain NCTC 9343 at a concentration

Figure 15



Reactions mediated by Co-enzyme B_{12} (after Lester Smith, 1965)

of 7.4 µg/ml. Meynell and Meynell (1970) suggested that DL isomers of amino acids were usually satisfactory for testing the growth requirements of microorganisms. In the present study it was found that DL methionine could not stimulate the growth of the three test strains of B. melaninogenicus ss. asaccharolyticus at concentrations of 7.4, 74 or 740 µg/ml in either ½PPY or PPY media. The concentrations used were selected in an attempt to allow for substantial variations between the B. melaninogenicus strains and the B. fragilis strains. Growth was obtained at each of the concentrations tested.

In the course of later gas-liquid chromatographic studies on strains of B. melaninogenicus and related species it was found that fatty acid production of the B. melaninogenicus ss. asaccharolyticus strain NCTC 9337 in ½PPY and PPY media was significantly affected by the addition of vitamin B_{12} at 1×10^5 pg/ml. Growth and propionic acid production were stimulated by cyanocobalamin but the production of n-butyric acid was partially inhibited. Varel and Bryant (1974) reported that the B. fragilis strain NCTC 9343 required the vitamin at a concentration of 1×10^2 pg/ml for good growth in a defined medium. To this author's knowledge the minimum growth requirements of B. melaninogenicus ss. asaccharolyticus strains have not been determined and there does not appear

to be any previous evidence in the literature of the pronounced effects that high concentrations of the vitamin can have on the growth and metabolism of these strains. Further studied in defined media are indicated.

The variation noted in the response of the test strains of B. melaninogenicus ss. asaccharolyticus to high doses of the vitamin, and the absence of any stimulation observed on some occasions, may have been due to the use of complex media and the fact that the strains could not be reliably starved of B_{12} before testing. Attempts to determine the baseline levels of vitamin B_{12} in both freshly prepared and stored BM, PPY and $\frac{1}{2}$PPY media by microbiological or radioisotopic dilution assays were not successful, possibly as a result of interfering substances in the media (Mr R. Samson, personal communication). The unreliability of the stimulation noted prevents the use of this observation as a diagnostic aid to identification at present.

Gas-liquid chromatography in the identification of Gram negative anaerobic bacilli

All the 185 Gram-negative, non-sporing, obligate anaerobes studied were identified to species or sub-species level by simple morphological, cultural, biochemical,

tolerance and antibiotic resistance tests (see Duerden et al., 1976). All isolates were correctly identified to genus level by independently performed GLC analyses of the short-chain fatty acids produced in glucose-free or glucose-enriched broth cultures. This study confirms that presumptive identification of genus can be made on the basis of Gram stain, cultural morphology and fermentation products (Holdeman and Moore, 1974), and that GLC data must be supplemented with other test data in order to further classify the Bacteroides and Fusobacterium genera.

Essentially similar fatty acid profiles were obtained with test strains of B. ovatus and B. fragilis and with reference strains of B. fragilis representing the five B. fragilis subspecies now accorded species rank (Cato and Johnson, 1976). The major products were succinic and acetic acids with traces of lactic and propionic acids and occasional strains produced traces of iso-valeric and iso-butyric acids. Moreover, there were further problems when pigment production was not known. All the B. fragilis-like strains tested produced similar fatty acids to those produced by the saccharolytic B. melaninogenicus strains; there were quantitative differences and minor qualitative differences, but none were of real discriminatory value at species or subspecies level. Furthermore,

B. oralis could not be differentiated from *B. melaninogenicus* ss. *melaninogenicus* nor *B. corrodens* from *B. melaninogenicus* ss. *asaccharolyticus*. The status of *B. oralis* (Loesche *et al.*, 1964) as a separate species is currently debated (ICSB Minute, in press). The strains may be non-pigmented variants of *B. melaninogenicus* ss. *melaninogenicus* (Harding *et al.*, 1976; Holbrook *et al.*, in press). The six *B. ochraceus* strains were not distinguished with certainty from the *B. melaninogenicus*-*B. oralis* group. The two saccharolytic subspecies of *B. melaninogenicus* produced succinic and acetic as major products in all of the test media, but production of lactic acid was variable, and there were frequently minor amounts of propionic, *iso*-valeric and *iso*-butyric acids; *n*-butyric acid was not produced. These strains were clearly distinct from the asaccharolytic subspecies which, in glucose-enriched or glucose-free media, typically produced moderate to large quantities of acetic, lactic and *n*-butyric acids and minor amounts of propionic, *iso*-butyric and *iso*-valeric acids; some produced succinic acid. *Fusobacterium* isolates typically produced acetic and *n*-butyric acids with traces of lactic and propionic acids, but did not produce *iso*-butyric or *iso*-valeric acid. The only significant products of the *Leptotrichia* isolates were moderate amounts of lactic and acetic acids. These

conclusions are summarized in table 31 .

The use of aqueous, semi-quantitative GLC analyses of the fermentation products of a wide range of reference and clinical strains of bacteroides revealed the considerable biological variation that exists within recognized groups of related organisms. In the B. melaninogenicus-B. oralis group, strain to strain and culture to culture variation occurred despite careful standardization of methods and obliged us to follow the example of Holdeman and Moore (1972) and develop a scoring system that took account of these variations but allowed recognition of meaningful differences between strains that might be useful in identification.

Shah et al. (1976) recently compared the biochemical properties of 45 B. melaninogenicus strains and reported differences in the fatty acid profiles of the two saccharolytic subspecies. Lactic and propionic acids were not detected in BM broth cultures of either subspecies. No B. melaninogenicus ss. melaninogenicus strains was found to produce iso-butyric acid but most ss. intermedius strains produced traces of this acid. The present results differ from those of Shah et al. (1976) who also noted discrepancies between their findings and those of Holdeman

Table 31 : Typical fermentation patterns in the family Bacteroidaceae

Species and subspecies	Relative amount of the stated acid* produced						
	A	P	iB	B	iV	L	S
B. fragilis-like strains	++ †	tr	(tr) ø	0	(tr)	tr	++
B. melaninogenicus ss. intermedius	+ - ++	0 - tr(+)	0 - +	0	0 - +	tr - ++	++
B. melaninogenicus ss. melaninogenicus	+ - ++	0 - tr(+)	0 - tr	0	0 - +	0 - ++	+ - ++
B. oralis	+ - ++	0 - tr(+)	0 - (tr)	0	0 - tr	0 - ++	+ - ++
B. ochraceus	+ - ++	0 - +	0	0	0 - (tr)	0 - (tr)	+ - ++
B. melaninogenicus ss. asaccharolyticus	+ - ++	+	(tr) - +	+ - ++	+	++	0 - +
Fusobacterium spp.	+ - ++	+	0	++	0	tr	0 - tr
Leptotrichia buccalis	tr - ++	0 - (tr)	0	0	0	++	tr

* see footnote table 18

For concentration ranges see table 25 (footnote)

† One reference strain each of B. distasonis and B. vulgatus produced + value only

ø( ) Occasional strains only

— Indicates the most typical result likely to be of discriminatory value.

and Moore (1972) and Lambe Jr. (1974). In the present author's experience the detection of < 5 µmols per ml of lactic or succinic acid in the aqueous volatile analyses was unreliable and methylation was necessary. Other small differences in the cultural or chromatographic procedures may account for other variations between our results and those of Shah et al. It was considered that the occurrence of such discrepancies in the determination of minor products, precludes the use of such small differences for subspecies identification or taxonomic differentiation of strains without support from other tests. The findings are in agreement with those of Moore (1970) who stated that the major fatty acid products are stable characteristics and it seems apparent that these products are consistently and reliably detected by different groups of workers.

The observation that vaginal isolates of saccharolytic B. melaninogenicus strains produced more lactic and succinic acids than oral or faecal strains of the same subspecies is interesting. Insufficient numbers were examined to draw definite conclusions but it is tempting to suggest that a population difference may exist between the intestinal and vaginal strains and that biotyping might be possible.

The BM medium of Williams et al. (1975), similar to

the isolation medium of Schaedler, Dubos and Costello (1965) proved to be an excellent culture medium and, with meat particles added, it is a useful maintenance medium for a wide range of anaerobic bacteria. It was considered that the high acetic acid content of BM medium, contributed by Trypticase, limited its value for our GLC studies and it was used only briefly in a preliminary series with freshly isolated oral strains.

The effect of restricted changes in the growth medium on fatty acid production was evaluated with five reference strains of the B. melaninogenicus-B. oralis group in six Proteose peptone media that did not contain Trypticase. Each strain produced a characteristic range of acids that was consistent in all the media except for the putative B. oralis strain NP 333 that produced traces of iso-valeric and lactic acids in glucose-free media only. Glucose in general affected the amounts but not the range of acids produced by saccharolytic strains. It did not affect acid production by the single asaccharolytic strain NCTC 9337. Similar observations have been reported previously (Werner, Pulverer and Reichertz, 1971; Werner, 1974; Mayhew, Onderdonk and Gorbach, 1975). Such observations support the general conclusion of Holdeman and Moore (1975) that chromatographic analyses could be run on any medium.

The choice of fully supplemented PPYSG medium, that contained Proteose peptone, 2.0% serum and 1.0% glucose, for definitive GLC studies was based on various considerations: (1) PPY media generally gave the best growth rates of a wide range of strains and the addition of serum enhanced the growth of some strains without affecting the fatty acid profiles. The author was concerned to achieve rapid growth wherever possible, to minimise incubation times and facilitate rapid identification of the isolates. (2) Many clinical isolates are likely to be saccharolytic strains that utilise glucose preferentially and grow more rapidly on this substrate. Asaccharolytic strains may show a slightly increased lag phase in glucose enriched media (Lev and Milford, 1975) but in these studies their fatty acid profiles were not affected. Where glucose is the preferred substrate for energy production the metabolism of it will generally cause catabolite repression of amino acid utilisation, but Loesche and Gibbons (1968) and Wahren and Gibbons (1970) have shown that fermentation of amino acids by some strains of F. nucleatum and B. melaninogenicus is not repressed by glucose. It seemed reasonable, therefore, to include glucose in the medium. The growth and fatty acid production of the B. melaninogenicus ss. asaccharolyticus strain NCTC 9337 was significantly affected by vitamin B_{12}

at a concentration of 1×10^5 pg/ml. This finding was discussed earlier in the context of the growth studies on vitamin B_{12}.

The GLC approach used in this study provided quantitative data useful in identification of the isolates and there is a high degree of correlation with the other independently performed tests. GLC may expedite the identification of clinical isolates to generic level without reliance on many other observations and tests, but it is clear that some basic data are necessary, even at this level, and additional information is required for species or subspecies identification. The appeal of GLC techniques for clinical laboratories is likely to be increased with recent reports suggesting that it is possible to diagnose the presence of anaerobes in clinical specimens by direct analyses on pus or other material (Gorbach et al., 1976; Phillips, Tearle and Willis, 1976; Bricknell, 1976). However, this calls for skilled and experienced staff able to monitor and interpret daily analyses. There must also be a sufficient number of specimens yielding anaerobes to justify the capital outlay for the equipment. At present, this author considers that if clinical microbiologists are encouraged to use existing GLC facilities available in reference laboratories, they could contribute to the

further refinement and development of identification procedures and to clarification of the remaining areas of confusion in the taxonomy of the anaerobic bacteria.

Identification and characterisation of the Bacteroidaceae

The routine identification of clinical isolates from the family Bacteroidaceae at present relies on relatively simple tests of phenotypic characteristics. In the VPI system (Holdeman and Moore, 1972), preliminary identification of species is accomplished by the use of a binary key utilising a combination of morphological and biochemical observations. Presumptive identifications are verified by comparing additional results with differential keys and tables based on a large number of phenotypic characteristics that correlate with genetic information when this is available (Moore and Holdeman, 1972). Clearly on some occasions, species and subspecies differentiation depends upon a few tests that reflect a very small portion of a cell's properties, but Moore and Holdeman (1972) state that in most cases the phenotypic characteristics are surprisingly accurate. The multifactorial approach of Duerden et al. (1976) was designed to overcome the difficulties occasionally produced by reliance on too few phenotypic tests for species and subspecies identification. This essentially practical approach placed stress on obtaining a pattern of results for each strain. The biochemical, tolerance and antibiotic

resistance test results were not intended for use in a sequential manner but as a combined group of tests.

The characterisation scheme of Duerden *et al.* was used to identify all the strains examined in the present study, and it was supplemented by a limited number of additional tests that were useful in differentiating a wider range of strains. Duerden and his colleagues had recognised certain key tests within the original complete scheme that they considered particularly useful for the presumptive identification of the major species and subspecies of the *B. fragilis*-like strains and for members of the *B. melaninogenicus* group. The key tests also enabled discrimination of these strains from organisms belonging to the related genera of *Fusobacterium* and *Leptotrichia* and assisted in the allocation of intermediate strains to the appropriate major group (Duerden *et al.*, 1976). The key characteristics for the species and subspecies tested by them are listed in table 32, reproduced with permission from Duerden *et al.* (1976).

The results obtained in my characterisation studies on some of the fifteen referred strains tested illustrated some of the difficulties confronting both research and clinical microbiologists in dealing with isolates from

Table 32 Scheme for the identification of Gram-negative anaerobic bacilli

	Result obtained in test																				
Pigment production†									−										+		
Antibiotic disc resistance tests:																					
neomycin (1000 μg)				R‡			S						S						S‡		
kanamycin (1000 μg)				R			R						S						R		
penicillin (1·5 units)				R			S						S						S‡		
rifampicin (15 μg)				S			S				R			S					S		
Tolerance tests:																					
taurocholate				+			I				I		+		I				I		
deoxycholate				I			I				I		+		I				I		
Victoria blue 4R		+			I	+		+	I		+	+	+		+				I		
ethyl violet		I			I	+		I	I		I	+	+		+				I		
Biochemical tests:																					
indole production	−	+	+	−	−		−	−		−	+	−		+	−		−	+	+		
digestion of gelatin	−	−	+	+	+		−	−		+	−	−		−	−		+	+	+		
hydrolysis of aesculin	+	+	+	+	+ or −		+	+		−	−	+		−	+		+ or −	−	−		
Fermentation of:																					
glucose	+	+	+	+	+		+	+		...§	...	...		...	...		+	+	−		
rhamnose	−	+	+	+	+		+	−		...	...	...		...	...		...	...	...		
trehalose	−	+	+	+	−		−	−		...	...	...		...	...		...	...	...		
mannitol	−	−	+	−	−		−	−		...	...	...		...	...		...	...	...		
	B. fragilis																*B. melaninogenicus*				
	ss. *fragilis*	ss. *thetaiotaomicron*	ss. *ovatus*	ss. *distasonis*	ss. *vulgatus*		*B. oralis*	*B. ochraceus*		*B. corrodens*	*F. necrophorum*	*F. necrogenes*		*F. polymorphum*	*L. buccalis*		ss. *melaninogenicus*	ss. *intermedius*	ss. *asaccharolyticus*		

† Pigment production (black colonies) observed on lysed blood agar after anaerobic incubation for up to 7 days.
‡ Occasional strains may give anomalous results.
§... Results of these tests are not of primary importance in the identification of these species.
Note: This gives sets of results that distinguish the species. The scheme should not be regarded as a sequential key.

reproduced from Duerden *et al.* (1976) with permission.

such a diverse and heterogeneous group as the family Bacteroidaceae. Confusion and lack of certainty with regard to the taxonomic status of some recognised species and subspecies still creates problems in naming fresh isolates. Difficulties are produced by the occurrence of intermediate strains in a continuum of variants that do not demonstrate characterisation patterns identical with those of accepted reference strains of recognised species and subspecies (Holdeman and Moore, 1974). Variations in the results obtained by different groups of workers testing accepted reference strains also contribute to the problems.

Thus none of the six referred strains thought to be representatives of the species B. oralis (Loesche et al., 1964) exactly fitted the characteristics previously described for this species by Holbrook (1976) and Holbrook et al. (1977). The strains closely resembled the previous descriptions but showed what were regarded as mainly minor differences in up to four key characteristics. Four of the putative B. oralis strains, the VPI strains 9958 and 7880 and the WAL strains 3030 and 3281, were resistant to the antibiotic neomycin in 1000 µg disks. The earlier studies had indicated that the majority of B. oralis strains (14/15) were sensitive to this concentration of neomycin. Apart from the absence of pigmentation in LBA plates, these

strains showed close similarities to the pigmented *B. melaninogenicus* ss. *melaninogenicus* strains listed in the table. This finding also confirmed previous observations and it is relevant that Duerden *et al.* (1976) also found that 50/53 strains from this subspecies were sensitive to neomycin at 1000 µg.

Similarly, only one of the *B. oralis* strains, the VPI strain 5540, was resistant to penicillin at 1.5 units and this finding is consistent with the earlier observations indicating that only occasional strains of *B. oralis* or *B. melaninogenicus* are resistant to this antibiotic. Duerden *et al.* (1976) noted that a significant number of strains of *B. melaninogenicus* gave atypical results with one or more of the test antibiotics in their scheme but they considered that resistance tests with a selection of antibiotics was useful for group discrimination in a combined approach with tolerance and biochemical tests. They considered that the validity of the patterns given for the *B. melaninogenicus* ss. *melaninogenicus* group and certain other groups must await further confirmation because of inadequate representation in these groups. The present studies did not examine sufficient additional strains to assist significantly in this confirmation. Previous results had indicated that most strains of *B. oralis* and *B. melaninogenicus* ss.

melaninogenicus hydrolysed aesculin but in the present studies two strains, VPI 5540 and 7880, failed to attack this substrate. Hydrolysis of aesculin has also been considered a feature of these strains by other workers (Holdeman and Moore, 1972; Harding et al., 1976).

The close similarities of some non-pigmented strains of B. oralis to some strains of B. melaninogenicus ss. melaninogenicus has been noted by various groups of workers who have therefore questioned the continued acceptance of black pigment as a key taxonomic characteristic (Duerden, 1975; Harding et al., 1976; Holbrook et al., 1977). Terada et al. (1976) and Sundqvist (1976) have separately suggested that there are close similarities between some strains of B. oralis, B. melaninogenicus ss. melaninogenicus and B. ruminicola. Sundqvist (1976) considered that the production of black-pigmented colonies was not a satisfactory basis for the discrimination of B. melaninogenicus strains from other non-pigmented species or subspecies. As the type strain of B. ruminicola did not grow in the thioglycollate and Robertson's CMB media used in the characterisation studies it was not possible to compare this strain directly with strains of B. oralis or B. melaninogenicus, but as Holdeman and Moore (1972) have considered that B. ruminicola strains could be distinguished from strains of B. oralis by the

fermentation of the pentose carbohydrates arabinose and xylose, it was still possible to check the test strains for these properties.

One putative B. oralis strain, the VPI strain 8906D, was a late fermenter of arabinose, but not xylose, in thioglycollate medium. When tested on two separate occasions the strain had not fermented after two days but had done so after incubation for seven days. The lowest pH recorded in the carbohydrate-enriched medium was 5.2. In terms of the criteria accepted by Duerden et al. (1976) as evidence of fermentation, the pH fall relative to the inoculated sugar-free control was 0.8 unit. As this isolate was designated a B. oralis strain by the VPI workers it clearly had not fermented this carbohydrate in their tests. The consistency of the present test results and careful purity checks encouraged the belief that technical error was not responsible for the discrepancy in the present study. It is difficult to assess the significance of this isolated result on a single strain; none of the other strains tested showed the same pattern.

Differences in the growth conditions, particularly the gaseous atmosphere and the media used, may partly explain the discrepancies between the present results

and those of Holdeman and Moore. The selection of different criteria as evidence of fermentation may also contribute. Holdeman and Moore (1972) noted a problem in fermentation testing with pentose sugars when they found that the pH of uninoculated, pre-reduced PY medium fell to 5.7 after 2 days in a carbon dioxide atmosphere, such as that used for gassing their media at the time of inoculation, and they suggested that a pH of 5.7 must be reached before it could be concluded that fermentation had taken place. In general these workers consider a pH fall in carbohydrate-enriched media to 5.5 to 6.0 is a weakly positive result and a fall to below 5.5 is regarded as evidence of strong acid production. There was no evidence that the 10% carbon dioxide used in the present studies could produce similar changes in uninoculated thioglycollate medium containing either arabinose or xylose.

The selection of different periods of incubation before final results are read can also lead to discrepancies in the results of fermentation tests performed by different groups of workers. Holdeman and Moore (1972) recommend that cultures be left to incubate only until good growth is observed on visual inspection. With an anaerobic jar technique, such a procedure is not feasible if cultures are not to be unduly disturbed during incubation.

Duerden *et al.* (1976) therefore selected two periods of incubation; cultures were read at two days and also after incubation for seven days. It might be considered that the somewhat imprecise methods recommended by Holdeman and Moore are likely to lead to the reporting of "false negative" results for late-fermenting strains, and conversely that the procedures of Duerden *et al.* may allow incubation to continue for too long before final results are read. Rutter (1970) suggested that prolonged incubation may lead to non-definitive results produced by the selection of a mutant population in the cultures. These and other aspects of fermentation were considered in the fermentation studies performed by the present author and the results of these studies will be considered later in the Discussion.

As it remains to be established whether the present separation of the species *B. oralis*, *B. ruminicola* and *B. melaninogenicus* ss. *melaninogenicus* is valid, further studies have been recommended (Anon., ICSB minutes of the 1976 meeting; *in press*). It appears, however, that the present separation of *B. oralis* and *B. ruminicola* largely on the results of two fermentation tests is undesirable; if the present grouping is eventually ratified it must be on the basis of more reliable tests reflecting stable characteristics.

The black-pigmented strain of B. melaninogenicus VPI 3300 referred to the Edinburgh team as the type strain of the proposed new subspecies levii (Holdeman and Moore) was typical of other strains of B. melaninogenicus in that it liquefied gelatin but was inhibited by bile salts and the dyes Victoria blue 4R, Ethyl violet, Gentian violet and Brilliant green; it was resistant to the antibiotics neomycin and kanamycin; and it was sensitive to penicillin and rifampicin at the concentrations stated in the Materials and Methods section of this thesis. It was a late weak fermenter of glucose and lactose but, unlike previously tested saccharolytic strains of the recognised subspecies B. melaninogenicus ss. melaninogenicus and ss. intermedius, it did not ferment maltose in the thioglycollate test medium. In addition, it did not ferment the pentose sugars arabinose or xylose that were omitted from the characterisation scheme of Duerden et al. (1976). Its fatty acid profile was typical of strains of B. melaninogenicus ss. asaccharolyticus, but unlike strains from that group it did not produce indole. The acids produced after incubation for 2 days in PPYSG medium included major quantities of lactic, n-butyric and acetic acids and minor quantities of propionic, iso-butyric and iso-valeric acids. It did not produce succinic acid. Later glucose utilisation studies showed that the strain

utilised less glucose over a period of seven days than representative strains of B. melaninogenicus ss. melaninogenicus or ss. intermedius, but it utilised more glucose in the same time than the type strain of B. melaninogenicus ss. asaccharolyticus, NCTC strain 9337. The results indicate clear differences between this strain and the previously recognised subspecies of B. melaninogenicus. The fermentation test results, the fatty acid profile, the aesculin, indole, gelatin, catalase and ox bile results are in agreement with the results of Holdeman and Moore and their co-workers as published in the latest additions to the VPI Manual (kindly supplied by Dr Jeremy Hardie). The VPI results are based however, on the examination of only four strains and the detailed characterisation of a larger number of strains is desirable to assist in the determination of the possible range of variation within the group and to establish with more certainty the degree of similarity or difference of these strains in comparison with each of the previously recognised subspecies. Such considerations may be particularly important with respect to glucose fermentation. Problems in fermentation testing and the results of glucose utilisation studies with some strains of B. melaninogenicus are considered separately in the final section of the thesis.

The apparent ability of these strains to ferment glucose and lactose and the absence of indole production appears to justify their exclusion from the subspecies B. melaninogenicus ss. asaccharolyticus. In addition the similarity of the fatty acid profiles is not necessarily indicative of a close relationship. The ability of the subspecies levii strains to produce significant quantities of n-butyric acid is evidence of significant utilisation of peptide or amino acid nitrogen (Barker, 1961) but this does not mean that some glucose fermentation cannot take place. It has been noted earlier that weakly fermentative strains of B. melaninogenicus are not prevented from utilising nitrogenous sources for energy production by glucose catabolite repression when the metabolite pool is not saturated with glucose intermediates (Wahren and Gibbons, 1970).

The strains of B. bivius, VPI numbers 6822 and 6318, and the B. disiens strains VPI 8057 and 7852, were clearly differentiated from other previously tested species or subspecies in the genus by comparison of the results obtained in the present studies with the reported findings of Duerden et al. (1976) and Holbrook (1976). These authors reported that of 105 reference and laboratory

isolates of B. fragilis and the closely related species of B. thetaiotaomicron, B. ovatus, B. distasonis and B. vulgatus, only five strains failed to ferment sucrose. Holdeman and Moore had reported similar findings in 1972 and more recently the same group of workers have, reported that none of the seventy eight strains of B. bivius (previously Bacteroides group PS) that they had examined from various worldwide sources had fermented this sugar (Anaerobe newsletter February, 1976). They concluded that this feature and the lack of growth in PYG medium enriched with 20% ox bile were the most significant differences between the strains of B. bivius and B. fragilis and its related species. These findings were confirmed in the present studies. It was found that the same criteria also separated strains of B. disiens from strains of the B. fragilis group. The test strains of both species were not stimulated by 20% ox bile (Oxgall, Oxoid) in PPYG medium and similarly they were inhibited by the bile salt sodium taurocholate at 0.5%. The strains of B. bivius and B. disiens were also distinguished from the same group by failing to hydrolyse aesculin and by the failure to ferment one or more of the sugars rhamnose, trehalose or mannitol. The results obtained were in agreement with recently published relevant additions to the VPI manual.

The strains of B. bivius and B. disiens were easily distinguished from strains of B. ochraceus by their inability to grow in air plus carbon dioxide and by their sensitivity to metronidazole an antimicrobial agent to which only obligately anaerobic bacteria are susceptible (Prince et al., 1969). The same strains were differentiated from strains of B. oralis by their resistance to neomycin at a concentration of 1000 µg and by their inability to hydrolyse aesculin and the absence of sucrose fermentation. Their inability to ferment rhamnose also distinguishes them from rhamnose fermenting strains of B. oralis. In addition the strains of B. disiens tested were distinguished from strains of B. oralis by the absence of lactose fermentation in thioglycollate medium.

The strains of B. bivius produced significantly higher quantities of lactic acid from two-day cultures in PPYSG medium than the test strains of B. disiens. In other respects the fatty acid profiles of the four strains were similar and typical of other carbohydrate fermenting species of Bacteroides. They produced major quantities of succinic and acetic acids with smaller quantities of propionic, iso-butyric and iso-valeric acids. None of the strains produced n-butyric acid.

The test strains of B. disiens were distinguished from the two strains of B. bivius by the absence of lactose fermentation and by the production of weak haemolysis on BA plates containing 5% human blood. The strains of B. bivius were also distinguished by the late fermentation of glucose. No other differences were apparent in the range of tests used.

Two colony variants were isolated on primary isolation plates inoculated from reconstituted freeze-dried cultures of the B. splanchnicus strain NCTC 10825, received from the National Collection of Type Cultures. Both variants and the other NCTC strain of the same newly-described species, NCTC 10826, were tested in the enlarged characterisation scheme. The species was originally described by Werner et al. (1975) as a saccharolytic Bacteroides species producing n-butyric acid. In the present study the morphology of both variant strains and the other strain was similar; they were all non-pigmented and non-sporing, pleomorphic, Gram-negative rods and coccobacilli. One colony variant of the NCTC strain 10825 was catalase producing and sensitive to penicillin in disks at a concentration of 1.5 units and was labelled NCTC 10825^{S}, (the S variant); the other variant, (R variant), NCTC 10825^{R}

did not produce catalase and was resistant to penicillin at the stated concentration. Werner et al. (1975) reported that all strains tested by them, including the two strains lodged with NCTC, were bacteriostatically inhibited by penicillin at concentrations of 5-40 μg/ml. It is possible that the culture submitted to the NCTC by Werner et al. (1975) contained both the S and R variants. It has been demonstrated in other bacterial groups that moderate penicillin resistant variants may arise in a population by a mutation (Hayes, 1968) but it might also be interesting to explore the possibility of a plasmid coding for β-lactamase production. The two variants may prove to be a good model for further study of the possible role of catalase in affecting oxygen tolerance or sensitivity of obligate anaerobes.

Other differences between the variants of NCTC 10825 were the partial inhibition of the S variant by the dye ethyl violet and the combined bile salts sodium taurocholate, 0.5% and sodium deoxycholate, 0.1%. The R variant was completely inhibited by ethyl violet and was not inhibited by the combined bile salts. In addition, the S variant fermented maltose after incubation for seven days. Neither the R variant of NCTC 10825 or the other NCTC strain NCTC 10826 fermented maltose in the present studies.

The NCTC strain 10826 alone fermented the pentose sugar xylose after incubation for seven days. Werner et al. (1975) had previously described both strains as fermenting arabinose but not xylose. These discrepancies in the fermentation tests, although minor, are further evidence of the variation that can occur in the results of different laboratories using different media and methods.

The NCTC strain of B. splanchnicus NCTC 10826 differed from the variants of the NCTC strain 10825 in its hydrolysis of aesculin and its failure to liquefy gelatin after incubation for fourteen days. Werner et al. (1975) did not report these reactions. The present studies confirmed that the strains produced large quantities of succinic, lactic, n-butyric and acetic acids with smaller quantities of propionic, iso-butyric and iso-valeric acids. The quantities of succinic, lactic and acetic acids were particularly high in comparison with typical results obtained from asaccharolytic strains of B. melaninogenicus ss. asaccharolyticus, to which the fatty acid profiles of the B. splanchnicus strains bear a qualitative resemblance. There should be no difficulty however in distinguishing representative strains of either group on the basis of other characteristics including the lack of pigmentation and the ready fermentation of glucose by strains of

B. splanchnicus.

B. splanchnicus strains are distinguished from strains of the B. fragilis group by the absence of growth stimulation by 20% ox bile, the production of significant amounts of n-butyric acid, and differing carbohydrate fermentation patterns. In the characterisation scheme of Duerden et al. (1976), four of the B. fragilis-like species, B. thetaiotaomicron, B. distasonis, B. ovatus and B. vulgatus typically ferment rhamnose whereas the strains of B. splanchnicus do not. Similarly, strains of B. thetaiotaomicron, B. distasonis and B. ovatus typically ferment trehalose and the B. splanchnicus strains do not. Variation in patterns of indole production and gelatin digestion render these two tests of no discriminatory value. Werner et al. (1975) reported that antisera prepared against the B. splanchnicus strains did not react serologically with antigens of strains representing the species B. fragilis, B. thetaiotaomicron, B. vulgatus and B. distasonis.

The clinical isolate Gnab 55s appeared to be an intermediate strain showing close similarities to the B. fragilis-like group. It differed from typical strains in the group by failing to be stimulated by the addition of 20% ox bile to PPYG medium. According to Holdeman and

Moore (1972) this finding is consistent with B. fragilis-like strains of no good fit. Its fatty acid profile was typical of a Bacteroides species producing significant quantities of succinic, lactic and acetic acids from two-day cultures in PPYSG medium; it produced smaller quantities of propionic and iso-valeric acids with a trace of iso-butyric acid, and it did not produce n-butyric acid.

According to the shortened characterisation scheme (table 31), its antibiotic resistance and tolerance patterns place it in the B. fragilis group of organisms. It differed biochemically from strains of B. vulgatus or B. distasonis by failing to digest gelatin after incubation for fourteen days and it was not inhibited by the dye Victoria blue 4R. As recent studies by other members of the Edinburgh team have indicated that the test results obtained with this dye may be affected by the method of addition of the dye to the test media (Dr B. Watt, personal communication), the latter result cannot be considered of real discriminatory value pending a full analysis of the problem.

The Gnab strain also fermented the pentose sugars, arabinose and xylose and although the media and methods

are different these results are consistent with Holdeman and Moore's (1972) findings for the B. fragilis-like strains. The strain differs significantly from other non-pigmented strains in the genus. The tolerance and antibiotic resistance patterns and its fermentation patterns are not similar to those of strains of B. oralis. Despite differences in test media and methods used, its fermentation pattern does not appear to be similar to the patterns reported by Holdeman and Moore (1972) for strains belonging to either of the recognised subspecies of B. ruminicola. The results of gelatin digestion, indole production and aesculin are similar to those obtained with B. ruminicola ss. ruminicola, but unlike these strains Gnab 55s ferments the sugars rhamnose, trehalose and maltose producing final pH values of 5.0 ± 0.1 in thioglycollate medium after incubation for only two days.

The Gnab strain differed from strains of B. disiens and B. bivius by growing in the presence of the bile salt sodium taurocholate, 0.5% and in the presence of the dye Victoria blue 4R; by fermenting the sugars sucrose, rhamnose, trehalose, arabinose and xylose; by hydrolysing dextran and aesculin and failing to digest gelatin. It differed from strains of B. splanchnicus by failing to

produce n-butyric acid in PPYSG medium, by the absence of haemolysis on BA plates, by failing to produce indole, and by fermenting sucrose, rhamnose, trehalose and arabinose under the test conditions used in these studies. It differed from all other strains tested in the present studies by the hydrolysis of dextran.

None of the strains examined gave positive results in the nitrate reduction tests on cultures in the indole-nitrite medium (BBL) that replaced the thioglycollate medium as the culture medium for this test in the present studies. Most test strains grew well in the medium and the positive and negative control cultures gave consistent clear results. The testing of nitrate reduction has been said to be particularly helpful in conjunction with other observations for distinguishing certain non-pigmented Bacteroides species including the separation of B. corrodens and B. praecutus strains from strains of B. putredinis and B. pneumosintes (Holdeman and Moore, 1972). The carcinogenic reagent 1-naphthylamine (BDH) gave no problems in routine use but a proper concern with the safety of laboratory workers suggested that an alternative reagent was desirable. A careful evaluation of the reagent 8-aminonaphthalene-2-sulphonic acid (Cleve's acid) used by Wideman, Citronbaum and Sutter (1977) has been planned. No evidence exists of a potential carcinogenic role for

this reagent and it is reported to be an adequate substitute for the previous reagent (personal communication, BDH Chemicals technical adviser).

Problems in fermentation testing examined with selected strains of B. melaninogenicus

Examples of inconsistencies in the present fermentation test results obtained with reference strains of different Bacteroides species compared with the results obtained by other workers were referred to earlier in this Discussion. These were attributed to differing culture methods, media or conditions, and to the selection of different periods of maximum incubation or even to different criteria of fermentation. Further evidence to support these contentions came from additional fermentation testing and glucose utilisation studies on selected clinical isolates of B. melaninogenicus that had presented difficulties in interpretation during earlier studies by members of the Edinburgh team.

The problems in fermentation testing affected a relatively small proportion of the clinical isolates of B. melaninogenicus tested in the characterisation scheme

by Holbrook et al. (1977) who reported results on 175 strains of B. melaninogenicus and related species; these were classified with the aid of the characterisation scheme of Duerden et al. (1976) extended by the gas chromatographic analyses of the fatty acid metabolic products of many of the strains. In the present study, a total of fourteen strains of B. melaninogenicus were selected for further study of their glucose fermentation and utilisation in complex media. The strains were all recent clinical isolates from human oral cavities, faecal or high vaginal specimens. They had all produced very similar patterns in the shortened characterisation scheme (table 31) except for differing results in the fermentation of glucose. They were all indole-producing and gelatin-digesting strains. Most seemed to be either non-fermentative or weak and late fermenters of glucose in thioglycollate medium and some showed significant discrepancies both in testing in this medium on successive occasions and when their reactions in this medium were compared with their fermentation reactions in other media.

On first testing the WPH strains, nos. 57, 118, 202, 210, 225, 228, 229 and 234 failed to produce a pH difference of 0.5 of a unit between the sugar free and glucose-enriched thioglycollate medium after incubation for seven days; in terms of the criteria established by

Duerden et al. (1976), these strains would be regarded as non-fermenting. However, only three of these eight non-fermenting WPH strains (57, 118 and 234) satisfied all the criteria and produced consistent fermentation test results that allowed their confident identification as strains of B. melaninogenicus ss. asaccharolyticus. Subsequent testing in the glucose utilisation experiments confirmed that these strains utilised no more glucose in ½PPYG medium than did the type strain of the subspecies asaccharolyticus, NCTC strain 9337. The WPH strains 225 and 234 did produce one discrepant result, each appearing to ferment in the PPYSG medium used for GLC analyses of the fatty acid metabolic products, but these were considered non-specific pH changes caused by the presence of serum in this medium. The WPH strain 225 was accepted as a strain of B. melaninogenicus ss. asaccharolyticus and was not selected for further study.

The identities of the WPH strains 202, 210, 228 and 229 remain in doubt; these strains showed some inconsistency by appearing to ferment in PPYSG medium and consistently failing to do so in thioglycollate medium. They also failed to ferment in Robertson's CMB medium or the ½PPYG medium that did not contain serum. The strains showed only light growth in the thioglycollate and ½PPYG

media after incubation for seven days but had grown well in the PPYSG and Robertson's CMB media. They did not utilise significant quantities of glucose in ½PPYG medium and were not stimulated to better growth by its addition to either thioglycollate or ½PPYG media; apart from the WPH strain 228 they did not ferment maltose, or any of the other carbohydrates tested, after incubation for seven days in carbohydrate-enriched cultures of thioglycollate medium. The strains had been tentatively identified as representative of B. melaninogenicus ss. intermedius on the basis of their other characterisation test results and particularly on the absence of n-butyric acid in their fatty acid products. The results showing apparent fermentation in PPYSG medium tended to support this conclusion but the findings with the WPH strains 225 and 234 suggested that serum could have a variable effect on the fermentation results in this medium. It is possible that the WPH strains 202, 210, 228 and 229 would produce different fermentation test results if they were tested in a medium supporting better growth than the thioglycollate or ½PPY media (see below).

The strains WPH 201, 214, 222, 223 and 226 all required prolonged incubation for seven days before apparent fermentation of glucose in thioglycollate medium. They produced significant quantities of n-butyric acid

in cultures in PPYSG medium and in all respects except the late fermentation of glucose they were indistinguishable from strains of B. melaninogenicus ss. asaccharolyticus. The strain WPH 222 also failed to ferment glucose in PPYSG medium after incubation for seven days on each of two occasions, and strain WPH 223 did so on one occasion. The strains WPH 201 and 226 produced pH differences of >1.0 unit between the glucose-free and glucose-enriched cultures and appeared to be more strongly fermentative in thioglycollate medium than the other strains which consistently produced pH differences between 0.5 and 0.9 of a unit in the same medium after seven days; at best, the latter could be considered late and weak fermenters of glucose. None of these test strains fermented maltose in thioglycollate medium.

At the time of first testing it was thought that some of these strains might represent a new subspecies of B. melaninogenicus and it was suggested that they required further study (Holbrook et al., 1977). Subsequently we received a culture of the proposed reference strain for the newly described subspecies B. melaninogenicus ss. levii, VPI 3300 and the present author was able to characterise this strain in the scheme and compare our clinical isolates with it. The results of the characterisation studies on the VPI strain are discussed

in an earlier section of this Discussion. The most significant differences between the clinical isolates at present under consideration and this strain was that the clinical isolates produced indole, and with the exception of WPH strain 223, they failed to ferment glucose in ½PPYG medium. In addition, all the clinical isolates required seven days to ferment glucose in thioglycollate medium, whereas the VPI strain fermented within four days. The amount of glucose utilised by some of the clinical strains was however similar to the amounts utilised by the VPI strain in the same medium. After seven days, the WPH strain 223 had utilised virtually the same amount of glucose as the VPI strain. Additional characterisation tests are required to establish with certainty the relationship of these strains; the difference in indole production suggests that the clinical isolates do not belong to the same subgroup as the VPI strain. It is possible that the clinical isolates are atypical strains of B. melaninogenicus ss. asaccharolyticus and that the fermentation test results are non-specific changes caused by prolonging the period of incubation beyond the time at which the results can be considered meaningful reflections of the specific saccharolytic capabilities of the organisms. This explanation seemed to be supported by the results obtained with the strain

WPH 98. On first testing this strain produced what appeared to be an inconsistent glucose fermentation test result. It produced a pH difference of 1.1 units in the thioglycollate cultures with this sugar alone, but in every other respect it was typical of a strain belonging to the subspecies B. melaninogenicus ss. asaccharolyticus, producing significant quantities of n-butyric acid in PPYSG cultures and failing to ferment glucose in these cultures after incubation for two or seven days. Replicate tests of seven-day cultures in thioglycollate medium confirmed the original result, but testing in Robertson's CMB, PPYSG and thioglycollate medium after incubation for four days failed to demonstrate evidence of fermentation. The original results with thioglycollate medium were not confirmed in later testing from a fresh freeze-dried culture opened for use in the glucose utilisation studies. Microscopic purity checks on all cultures in both series of tests failed to demonstrate a contaminant. In the glucose utilisation studies the organism behaved as a typical strain of B. melaninogenicus ss. asaccharolyticus, failing to ferment glucose in either thioglycollate or ½PPYG media after incubation for seven days, and it utilised glucose at low levels similar to that of the type culture of the subspecies.

Further evidence supporting the suggestion that incubation for seven days is unnecessary came from the glucose utilisation patterns of the five reference strains representing the four subspecies of B. melaninogenicus and from the earlier studies on some of these strains in the experiments showing the effect of the VPI balanced salts solution (Holdeman and Moore, 1972) on the growth of the different subspecies of B. melaninogenicus. In the glucose utilisation study the glucose-fermenting strains of B. melaninogenicus ss. melaninogenicus VPI 4196, the ss. intermedius strain NCTC 9336 and the ss. melaninogenicus strain ATCC 15930 (designated as B. oralis) had all utilised maximal amounts of glucose by the fourth day of incubation in ½PPYG medium. The B. melaninogenicus ss. levii strain VPI 3300 also showed maximal rates of utilisation after the same period of incubation but continued to utilise a small amount of glucose after that time. The growth curves prepared from duplicate anaerobic jar cultures of the NCTC strain 9336 and the VPI strain 4196 in the earlier studies indicated that maximum growth and the associated pH fall in glucose-enriched ½PPYG medium occurred within the first two to three days for these strains. In addition, the same studies showed that the asaccharolytic strain of B. melaninogenicus ss. asaccharolyticus NCTC 9337 produced

maximal levels of growth within three days in the same medium. These findings and observations on a wide range of clinical isolates representing the three recognised subspecies of B. melaninogenicus indicate that the maximum period of incubation for fermentation tests should be no more than five days. The choice of a five-day maximum should allow for strain to strain variations and avoid the type of anomalies found previously.

The amount of acid produced by fermenting bacteria in carbohydrate-enriched culture media varies with different bacterial groups and depends partly on the manner in which the sugar is metabolised (Meynell and Meynell, 1970); it can also be significantly affected by the starting pH of the medium (Simon, 1968 cited by Langworth, 1977). The poising of the culture conditions and the careful selection of an appropriate pH difference between the carbohydrate-free and the carbohydrate-enriched test medium that will be sensitive enough to indicate genuine weakly fermentative strains yet not produce "false positive" results from asaccharolytic strains is particularly important. The choice of a suitable pH difference is to some extent an arbitrary one but it should be selected to allow for culture to culture variation and must be confirmed by numerous observations

with reference and clinical isolates from the group of organisms to be identified. The problems reported in this thesis appear to have been caused by more than one factor but it is difficult to avoid the conclusion that some of the problems were caused by accepting an unduly small pH difference as evidence of fermentation. The detailed observations on an admittedly biased selection of clinical isolates and on a few reference strains suggest that some asaccharolytic strains can produce non-specific changes in thioglycollate medium up to values of approximately 1.0 unit. Other observations on a wide range of strains from the same species suggest that on average such changes may be as high as 0.6 of a unit in this medium (Mr R. Brown, personal communication). Rutter (1970) considered that non-specific changes of this order could occur in cultures of C. oedematiens in the Robertson's CMB medium containing 1% glucose that he used for fermentation tests on those organisms. It is likely that a change of the test medium will alter the extent of such non-specific changes; thus, if a new medium is chosen to replace the thioglycollate medium, this factor will require further consideration.

Some of the problems in fermentation testing noted may have been caused by mutants arising in the cultures.

Such mutants could give rise to rapid, delayed or weak reactions depending upon the frequency and timing of their expression. It might be interesting to investigate this possibility with a few of the selected strains by performing a fluctuation test similar to that of Lewis (1934) but it may prove difficult to develop a suitable synthetic medium, containing glucose as the sole source of carbon, that would grew these strains.

The growth studies reported in this thesis confirmed the requirement for Proteose peptones and enriched culture media for some asaccharolytic strains of B. melaninogenicus and showed that the growth of a wide range of B. melaninogenicus strains was significantly improved by the use of such media. Good growth in fermentation test media is essential for consistent results. This is particularly important if the present criterion for fermentation is to be retained. Only when there is good growth in both the sugar-free control cultures and in the carbohydrate-enriched medium can the determination of pH differences in the two media be considered meaningful. Rutter (1970) and Holdeman and Moore (1972) have also stressed the importance of obtaining good growth in fermentation media in which the production of acid is measured with a pH electrode. Good growth

in a culture medium that has an adequate supply of all the essential growth factors including trace minerals and vitamins is essential to ensure that the adaptive response of the inoculated cells to the added substrate is not impaired by any nutritional deficiency. It had been hoped that the $\frac{1}{2}$PPY/$\frac{1}{2}$PPYG media used in the glucose utilisation studies would prove satisfactory and might replace the thioglycollate medium at present used in the characterisation scheme, but the slow growth noted with some of the strains examined in these studies suggests that an even richer alternative must be chosen. Future studies could explore the possibility of using the BM-S medium tested in the growth studies or a different version of this type of medium containing smaller quantities of Trypticase.

Rutter (1970) suggested a possible difficulty in using rich media for fermentation tests when he suggested that the adaptive response of some strains could be delayed if the inoculated cells preferentially utilise substrates present in the basal medium. This suggestion has important implications for fermentation testing of nutritionally demanding organisms. It appears to suggest that in testing such strains no reliance can be placed on weak or delayed fermentation reactions because it would

not be possible to distinguish a delayed response from non-specific changes associated with prolonged incubation. Such difficulties raise the question of the usefulness of fermentation tests as a fine taxonomic tool. Other workers have recognised that there are problems in using fermentation tests for identification purposes; Schwabacher et al. (1947) reported the acidogenic nature of some B. melaninogenicus strains and considered that the amount of acid produced in the basal medium rendered fermentation tests impractical. Shah et al. (1976) have also commented on the acidogenicity of B. melaninogenicus ss. intermedius strains in particular and stated that, in their hands, strains of subspecies melaninogenicus and asaccharolyticus were less prone to produce significant amounts of acid at least in their version of BM medium. Their findings were confirmed in the present studies and the results in table 29 of the Results section, indicate that the three WPH strains 202, 228 and 229, thought to be representatives of B. melaninogenicus ss. intermedius, produced significant amounts of acid in the carbohydrate-free PPYS and Robertson's CMB media. Similar findings were noted on many strains tested during earlier characterisation studies. The production of acid in the basal medium appeared to be a strain characteristic

and although it occurred in thioglycollate medium it seemed to occur more frequently in the media supplemented with horse serum. Such findings underlined the importance of retaining the existing criterion of a pH difference between the carbohydrate-free and carbohydrate-enriched medium rather than adopting the procedure of simply monitoring the pH fall in carbohydrate-enriched media for the determination of fermentation as used by other workers (Holdeman and Moore, 1972; Shah et al., 1976). Fermentation testing in this group of organisms is clearly not without its difficulties but with careful standardisation of media, growth conditions and testing procedures and an awareness of the problems that can occur with a minority of strains it seems likely that it can still be useful in identification on the basis of a combined approach with other tests, particularly at the level of identifying clinical isolates. In diagnostic laboratories there is a requirement for simple and rapidly performed tests. Despite these conclusions there is still a need to continue the search for alternative approaches for the identification of the Bacteroidaceae at the clinical level and in the future increasing stress may be placed on relatively simple serological tests or on the differentiation of the subspecies and species by the use of tests reflecting

stable characteristics such as the electrophoretic separation of cellular proteins as determined by polyacrylamide electrophoresis (Strom et al., 1976), enzyme assays (Funderburk and Kester, 1975), the electrophoretic mobility of enzymes such as NAD-dependent malate dehydrogenase (Williams et al., 1975; Shah et al., 1976), or by some other approach involving unique structural or metabolic characteristics.

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APPENDICES

APPENDIX I

Methods of preparing the media and supplements described in the Materials and Methods section

Media used in these studies

BM broth (modified from Williams et al., 1975)

Trypticase (BBL)	1%
Proteose peptone (Oxoid)	1%
Yeast extract (Difco)	0.5%
Sodium chloride	0.5%

Adjust to pH 7.4, autoclave at 121°C for 15 min. and store at 4°C. Before use, steam for 30 min. and cool rapidly to 37°C. To the cooled medium add filter-sterilised aqueous solutions of:

Haemin	5µg/ml
Menadione	1µg/ml
L-cysteine hydrochloride	0.075%
Horse serum (Wellcome)	2%
4% sodium carbonate	0.04%

(The modifications from Williams et al. are the deletion of glucose and the replacement of bovine serum with horse serum.)

BM-CM broth

BM medium was prepared and fresh cooked meat particles (ox heart) were added before sterilisation to a depth of c. 2 cm in 10-ml tubes of the dispensed basal medium. Horse serum was not added to this medium.

Further modifications to BM medium tested in this study are detailed in table 4 in the main text.

Robertson's Cooked Meat broth (modified from Cruickshank et al., 1975).

Boil minced ox heart and dry the meat particles.
Dispense into test tubes to a depth of about 2 cm.
Add 10 ml of nutrient broth no. 2 (Oxoid)
To prepare the broth, dissolve 2.5 g of the powder in 100 ml distilled water. Autoclave the complete medium and store at 4°C. Prior to use, steam for 30 min. and cool rapidly to 37°C immediately before inoculation.

VL medium (as modified by Barnes and Impey, 1971, from the original recipe of Beerens et al., 1963).

Tryptone (Oxoid)	1.0%
Beef extract (Lab-Lemco, Oxoid)	0.3%
Yeast extract (Difco)	0.5%
NaCl	0.5%
glucose	0.25%
cysteine HCl	0.04%
agar (New Zealand)	0.06%

Adjust pH to 7.4; autoclave at 121°C for 15 min. and dispense in 19-ml quantities in 1-oz screw-capped McCartney bottles. Details of the haemin, liver and faecal extracts added to this medium are given in the text. For the solid VL agar plates, 1.2% New Zealand agar is added.

PY medium (Holdeman and Moore, 1972)

Bacto Peptone (Difco)	1%
Yeast extract	1%
Balanced salts solution	4 ml/100 ml

Adjust pH of basal medium to 7.2 dispense and autoclave. Before use, steam for 30 min., cool rapidly and add the filter-sterilised supplements:

Haemin	5µg/ml
Menadione	1µg/ml
Cysteine HCl	0.05%

Sodium carbonate is not added but is replaced by sodium bicarbonate in the balanced salts solution.

Balanced Salts Solution (Holdeman and Moore, 1972)

$CaCl_2$ (anhydrous)	0.2g
$MgSO_4$	0.2g
K_2HPO_4	1.0g
KH_2PO_4	1.0g
$NaHCO_3$	10.0g
NaCl	2.0g

Prepared as described by Holdeman and Moore (1972). For use in this study the solution was prepared fresh and added to the basal media prior to autoclaving. The solution could not be sterilised by autoclaving itself because some salts crystallised out of solution on heating.

PP3 medium for use in growth studies

Proteose peptone no. 3 (Difco)	1%
Yeast extract (Difco)	1%
Balanced salts solution	4 ml/100 ml

Adjust pH to 7.2, dispense and autoclave. Steam the medium for 30 min. prior to use and add:

Haemin	5µg/ml
Menadione	1µg/ml
Cysteine HCl	0.05%

½PPY medium used in growth studies and gas chromatography studies

Proteose peptone (Oxoid)	1%
Yeast extract	0.5%
NaCl	0.5%

Adjust pH to 7.2, dispense and autoclave. To the steamed cooled basal medium add:

Haemin	5µg/ml
Menadione	1µg/ml
Cysteine HCl	0.075%
Na_2CO_3	0.04%

PPY medium

Proteose peptone (Oxoid)	2%
Yeast extract (Difco)	1%
NaCl	0.5%

Prepared as for ½PPY medium and the same supplements added.

A number of versions of the ½PPY and PPY media were prepared in the course of the studies. ½PPYS and PPYS media in addition to the other ingredients contained 2% horse serum and ½PPYG and PPYG media contained 1% glucose. PPYSG medium contained 2% horse serum and 1% glucose.

PPLI medium

Proteose peptone no. 3 (Difco)	1%
Liver infusion (Oxoid)	1%
Balanced salts solution (Holdeman and Moore, 1972)	4 ml/100 ml

Adjust to pH 7.0, dispense and autoclave. Steam the basal medium for 30 min. prior to use, cool rapidly and add filter-sterilised solutions of:

Haemin	5µg/ml
Menadione	1µg/ml
Cysteine HCl	0.075%

PPLD medium

Proteose peptone no. 3 (Difco)	1%
Liver digest (Oxoid)	1%
Balanced salts solution	4 ml/100 ml

Prepared, dispensed and supplemented as for PPLI medium.

PLY medium

Yeast extract (Difco)	0.3%
Beef extract (Lab-Lemco, Oxoid)	1.0%
Bacto peptone (Difco)	1.0%
NaCl	0.5%

Prepared, dispensed and supplemented as above.

Nitrate broth (BBL - Indole Nitrite medium)

Trypticase (BBL)	2%
Na_2HPO_4	0.2%
Dextrose	0.1%
Agar (Oxoid ion agar no. 2)	0.1%
Potassium nitrate	0.1%

Adjust pH to 7.2 ± 0.1. Boil for 1 min. to dissolve agar. Dispense in 10-ml amounts and autoclave. Steam the medium for 30 min. prior to use and cool to 37°C and add:

Haemin	5µg/ml
Menadione	1µg/ml

Thioglycollate medium (modified from Loesche, Socransky and Gibbons, 1964) for use in characterisation studies

Thioglycollate medium without dextrose or indicator (BBL)	24g
Yeast extract (Oxoid L21)	0.25%
Sodium succinate	0.25%
Distilled water	1 litre

Dissolve by boiling for 1 min. Dispense in 10-ml amounts and autoclave. Steam for 30 min. prior to use, cool to 37°C then add filter-sterilised solutions of:

Haemin	5µg/ml
Menadione	1µg/ml

Trypticase Soy broth (BBL) and Nutrient broth no. 2 (Oxoid) for use with the redox potential electrode were prepared according to the manufacturer's instructions.

Supplements

Haemin solution

Haemin (BDH)	50 mg
N/10 NaOH	10 ml

Dissolve haemin in NaOH and add 90 ml distilled water; membrane filter and store at 4°C.

Menadione solution (Barnes and Impey, 1971)

Dissolve 10 mg menadione (Sigma) in 1 ml ethyl alcohol. Add distilled water to 100 ml. Membrane filter and store at 4°C in dark bottles. For routine purposes the stock solutions of haemin 500 µg/ml, and menadione 100 µg/ml, were mixed in equal parts and added as a single combined solution to the cooled, autoclaved or pre-steamed media to give a final concentration of haemin 5 µg/ml, and menadione 1 µg/ml.

Cysteine hydrochloride solution

Dissolve 3.75 g of cysteine hydrochloride (BDH) in 100 ml of distilled water; membrane filter and store at 4°C. The solution is discarded and replaced if re-crystallisation occurs.

All other supplements used in these studies were similarly prepared and sterilised. Membrane filtration was performed with 0.22 µ Millipore filters.

APPENDIX II

Names and addresses of suppliers of materials

The American Type Culture Collection: 12301 Parklawn Drive, Rockville, Md. 20852, U.S.A.

Baird and Tatlock Ltd: Romford, Essex RM 11HA, England.

Baltimore Biological Laboratories (BBL) Ltd: York House, Empire Way, Wembley, Middlesex HA 90PS, England.

BDH Chemicals Ltd: Broom Road, Poole, Dorset BH12 4NN, England.

Belmont Instruments: 2 Clairmont Gardens, Glasgow G3 7LW, Scotland.

British Oxygen Company (BOC) Ltd: Special Gas Division, Deer Park Road, London SW19 3UF, England. and BOC Ltd: Seafield Road, Leith, Edinburgh, Scotland.

Calbiochem Ltd: 79/81 South Street, Bishops Stortford, Hertfordshire CM23 3AL, England.

Chromatography Services Ltd: 23 Old Chester Road, Lower Bebington, Wirral, Merseyside L63 7LA, England.

Difco Laboratories: West Molesey, Surrey, England.

Englehard Industries Ltd: Valley Road, Cinderford, Gloucestershire GL14 2PB, England.

Geo. T. Gurr and Sons Ltd: P.O. Box 1, Romford, Essex RM1 1HA, England.

Hawkesley Ltd: 12 Peter Road, Lancing, Sussex, England.

Honeywill and Stein Ltd: Mill Lane, Carshalton, Surrey, England.

Hopkins and Williams Ltd: Chadwell Health, Essex, England.

Koch Light Laboratories Ltd: Colnbrook, Bucks, England.

Mast Laboratories Ltd: Liverpool, England.

MSE/Fisons Ltd: MSE Scientific Instruments, Crawley, Sussex, England.

National Collection of Type Cultures: Central Public Health Laboratory, Colindale Avenue, London, England.

Oxoid Ltd: Wade Road, Basingstoke, Hampshire RG24 0PW, England.

Phase Separations Ltd: Deeside Industrial Estate, Queensferry CH5 2LR.

Pye Unicam Ltd: York Street, Cambridge, England.

Sigma (London) Chemical Co. Ltd.: Norbiton Station Yard, Kingston-upon-Thames, Surrey KT2 7BH, England.

Virginia Polytechnic Institute and State University: Anaerobe Laboratory, Blacksburg, Va 24060, U.S.A.

Wellcome Research Laboratories: Beckenham BR3 3BS, England.

Carl Zeiss Ltd: Carl Zeiss House, 31-36 Foley Street, London W1P 8AP, England.

APPENDIX III

Report to the International Committee of Systematic Bacteriology (I.C.S.B.) Taxonomic Subcommittee on Gram-negative Anaerobic Rods.

(N.B. see tables attached in pocket inside back cover).

REPORT TO THE I.C.S.B. TAXONOMIC SUB-COMMITTEE ON GRAM-NEGATIVE ANAEROBIC RODS

B.I. Duerden
W.P. Holbrook
A.G. Deacon
R. Brown and
J.G. Collee

Microbial Pathogenicity Research Laboratory,
Department of Bacteriology,
Edinburgh University Medical School,
Teviot Place,
Edinburgh EH8 9AG.

INTRODUCTION

As part of the collaborative investigation instigated by the Taxonomic Sub-committee on Gram-negative anaerobic rods, 40 strains of Bacteroides melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus were subjected to a series of tests. These strains included the 12 referred to us by the sub-committee, 6 strains referred by other colleagues and 22 strains isolated from subgingival dental plaque in our laboratory. This work forms part of our continuing studies with B. melaninogenicus and related organisms.

MATERIALS AND METHODS

Organisms. Bacteroides melaninogenicus ss. melaninogenicus strains WAL 2721* and WAL 2724* (Dr S.M. Finegold), GUI 1011* and GUI 1034* (Dr K. Ueno), VPI 4196 (Dr E. Cato, V.P.I.) and 15 strains isolated from subgingival dental plaque in our laboratory.

Bacteroides oralis strains VPI 7570A* and VPI 5832* (V.P.I., Blacksburg, Va.), J1*, 7CM* and 30* (Dr E. Sharpe), ATCC 15930* (American Type Culture Collection), NP 333 (Dr J.M. Hardie, London Hospital Medical College), and 7 strains isolated from subgingival dental plaque in our laboratory.

Bacteroides ochraceus (Ristella ochraceus) strains 1956C* and 2467B* (Dr M. Sebald), VPI 2845 (V.P.I., Blacksburg, Va.), 10, 79B and 73 (Dr W.H. van Palenstein-Helderman, Preventive Dentistry Dept., University of Utrecht, Netherlands).

* Strains referred by the I.C.S.B. Taxonomic Sub-committee.

Characterization of Strains

The 40 test strains were subjected to the following series of morphological, biochemical, tolerance, and antibiotic disk resistance tests (for details of media and methods, see Duerden et al., 1976) and to gas-liquid chromatographic (G.L.C.) analysis of their short chain fatty acid metabolic products of glucose.

Morphological and biochemical tests: Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen sulphide production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose, and mannitol.

Tolerance tests: Growth in the presence of (i) the bile salts sodium taurocholate, sodium deoxycholate, and the combination of sodium taurocholate plus sodium deoxycholate, and (ii) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet.

Antibiotic disk resistance tests: Resistance to disks containing neomycin 1000 μg and 10 μg, kanamycin 1000 μg and 30 μg, penicillin 1.5 units, methicillin 10 μg, erythromycin 60 μg, colistin 10 μg, rifampicin 15 μg, lincomycin 2 μg, clindamycin 2 μg, bacitracin 0.1 unit, vancomycin 15 μg, chloramphenicol 10 μg, tetracycline 10 μg and metronidazole 5 μg.

G.L.C. analysis of short-chain fatty acids.

Medium: The strains were grown for 48 h in a glucose-containing medium (PPYSG) containing: Proteose peptone (Oxoid) 2%; yeast extract (Difco) 1%; and NaCl 0.5%. Filter-sterilized solutions of the following heat-labile supplements were added aseptically to the (cooled) autoclaved basal medium (pH 7.4) to give final concentrations of: inactivated horse serum, 2%; glucose, 1%; haemin, 5 µg/ml; menadione 1 µg/ml; cysteine hydrochloride, 0.75%; and Na_2CO_3, 0.04%. The pH at inoculation was 7.1 ± 0.1. The inoculum was one drop (0.02 ml) of a 48-h culture in cooked-meat broth.

Chromatograph. A Pye-Unicam series 104 gas chromatograph fitted with heated injection ports and dual flame-ionization detectors was operated isothermally at 190°C with a detector temperature of 250°C. The carrier-gas was oxygen-free nitrogen at a flow-rate of 35 ml/min. and the hydrogen flow-rate of both detectors was adjusted for optimum sensitivity. The instrument was fitted with two identical glass columns (1.5 m x 4 mm) containing Chromosorb 101 (Johns-Manville Corp., U.S.A.; supplied by Gas Chromatography Services Ltd., 23 Old Chester Road, Lower Bebington, Wirral, Merseyside, L63 7LA). Columns were packed in the laboratory. The recorder was a Servoscribe 1S model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow, G46 7PA) set at the 10 mV range with a recorder speed of 120 mm/h.

Analysis. The procedures used were derived from those of Carlsson (1973).

(a) Volatile acids. Cultures were acidified with 50% sulphuric acid to pH 2.0 and a 0.6 µl sample of cell-free supernatant was injected directly on to the analysing column without further pre-treatment or extraction. Contamination of the top 2-3 cm of the column occurred during use and required periodic replacement with fresh polymer. The attenuation setting was 2×10^{-2} at the x 1 range setting.

(b) Non-volatile acids. The acidified culture supernates were methylated according to the method of Holdeman and Moore (1972) and a 0.6 µl sample of the chloroform extract was injected on to the column under the same analysis conditions as for the volatile acids but with an attenuation setting of 5×10^{-2}.

Lactic and succinic acids were detected qualitatively in the analysis of volatile acids, but this was confirmed quantitatively by the methylation procedure.

Standards. Single and combined 0.01 M aqueous standards of the volatile acids were used to establish absolute and relative retention times; 0.02 M standards were used in the analysis of the non-volatile acids. Samples of uninoculated (sterile) PPYG medium were included as controls in every batch of each of the two types of analysis.

A PPYG control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as a routine each day.

The approximate concentration values of acids for test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. Results were recorded as follows:

<u>Volatile acids</u>.	Concentration value	> 10 μM/ml:	++
	" "	1.1 - 10 μM/ml:	+
	" "	0.2 -1.0 μM/ml:	tr (trace)
	" "	< 0.2 μM/ml:	-
<u>Lactic and succinic acids</u>.			
	Concentration value	> 20 μM/ml:	++
	" "	10 - 20 μM/ml:	+
	" "	1 - 9 μM/ml:	tr
	" "	< 1 μM/ml:	-

RESULTS

The results of morphological, biochemical, tolerance and antibiotic disk resistance tests with the 40 test strains are shown in table 1. The proportion of strains that gave each result are shown in table 2, and the results obtained with the 12 strains referred by the I.C.S.B. Taxonomic Sub-committee are shown in table 3.

PRELIMINARY ASSIGNMENT OF TEST STRAINS TO GROUPS

The test strains submitted as B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus were divided into three groups for this analysis:

(i) Strictly anaerobic strains that produced black or brown pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to one group labelled B. melaninogenicus ss. melaninogenicus (23 strains).

(ii) Strictly anaerobic, non-pigmented strains - B. oralis (11 strains). Three strains (ATCC 15930*, VPI 7570A, and 30) were referred to us as strains of B. oralis but produced black or brown pigmented colonies and are included in our B. melaninogenicus group (above).

(iii) Non-pigmented strains that were able to grow in 10% CO_2 in air - B. (Ristella) ochraceus (6 strains).

* See Holbrook and Duerden (1974).

MORPHOLOGY

Microscopic appearance. All strains were Gram-negative bacilli and many were pleomorphic. B. melaninogenicus ss. melaninogenicus and B. oralis strains were predominantly cocco-bacilli, occasionally arranged in short chains. B. ochraceus strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology. The colonies of B. melaninogenicus ss. melaninogenicus were 1-2 mm diameter, round, convex and opaque. After incubation for 48 h, they were typically light grey, becoming brown after further incubation. The pigmentation varied between strains from light brown to almost black.

Colonies of B. oralis were 1-2 mm diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 days, the colonies of some strains (including VPI 5832) became light brown and were difficult to distinguish from the lighter-pigmented strains of B. melaninogenicus ss. melaninogenicus.

B. ochraceus strains typically produced two colony types: (a) 1 mm diameter, round or with an irregular edge, smooth, opaque, and blue-grey; (b) 1 mm diameter, rhizoid, granular and ochre in colour.

TOLERANCE TESTS

(a) Bile salts. All strains of B. melaninogenicus ss. melaninogenicus and B. ochraceus were completely inhibited by the test concentrations of sodium taurocholate, sodium deoxycholate, and the combination of the two salts. Ten strains of B. oralis were also inhibited, but the growth of strain 7CM was not inhibited by either of the bile salts alone or by the combination.

(b) Dyes. All test strains were completely inhibited by the test concentrations of gentian violet and brilliant green, and only B. oralis strain 7CM was able to grow in the presence of ethyl violet. Twenty-one strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis (including VPI 5832) and 2 of B. ochraceus (strains 1956C and VPI 2845) were completely inhibited by Victoria vlue 4R; however, 2 strains of B. melaninogenicus ss. melaninogenicus (WAL 2724 and one laboratory isolate), 5 strains of B. oralis (7CM, J1, NP333 and 2 laboratory isolates), and 4 strains of B. ochraceus (2467B, 10, 79B and 73) were able to grow in the presence of Victoria blue 4R.

BIOCHEMICAL TESTS

None of the test strains produced indole, catalase, or oxidase. All test strains produced acid from glucose, lactose, sucrose and maltose, but not from trehalose or mannitol.

Production of H_2S. Most strains produced H_2S, but some produced only small amounts and production by 2 strains of B. melaninogenicus ss. melaninogenicus and 2 strains of B. oralis (all laboratory isolates) was not detected.

Haemolysis. All strains of B. melaninogenicus ss. melaninogenicus produced some degree of haemolysis on human-blood agar. In most cases this was a zone of complete haemolysis around the colonies, but a number of strains, including WAL 2721, WAL 2724, GUI 1034 and VPI 7570A produced only a small zone of incomplete haemolysis. Two strains of B. oralis (J1 and 7CM) produced clear zones of complete haemolysis, 7 strains (including VPI 5832 and NP333) produced small zones of incomplete haemolysis and 2 laboratory isolates were non-haemolytic. None of the B. ochraceus strains were haemolytic.

Rhamnose fermentation. Six strains of B. oralis (including J1, 7CM, VPI 5832 and NP333), but no strains of B. ochraceus and only one laboratory isolate of B. melaninogenicus ss. melaninogenicus produced acid from rhamnose.

Aesculin hydrolysis. Twelve of the 23 strains of B. melaninogenicus ss. melaninogenicus (strains ATCC 15930, GUI 1011, GUI 1034, VPI 7570A, 30 and 7 laboratory isolates), all of the test strains of B. oralis except 7CM, and all strains of B. ochraceus except 1956C hydrolysed aesculin.

Dextran hydrolysis. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, ATCC 15930, WAL 2724 and 30), all strains of B. ochraceus, and 5 strains of B. oralis (including NP333) hydrolysed dextran.

Gelatin digestion. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, ATCC 15930, GUI 1011 and GUI 1034), but only 3 laboratory isolates of B. oralis, digested gelatin disks. The other 8 strains of B. oralis and all 6 strains of B. ochraceus failed to digest the disks.

Lipase production. Strain 30 and seven other strains of B. melaninogenicus ss. melaninogenicus produced a lipase effect. This effect was not produced by any strain of B. oralis or B. ochraceus.

ANTIBIOTIC-DISK-RESISTANCE TESTS

All test strains were sensitive to erythromycin, rifampicin, lincomycin, clindamycin and chloramphenicol. They were all resistant to neomycin (10 μg), kanamycin (1000 μg and 30 μg) and bacitracin. Two strains, nos. 30 and 7CM, were resistant to neomycin (1000 μg), but all other strains were sensitive. All strains except ATCC 15930 were resistant to vancomycin. Five of the 6 strains of B. ochraceus were resistant to metronidazole; strain VPI 2845 was sensitive to metronidazole, but produced a smaller zone around the disk than the strictly anaerobic bacteroides organisms. All

strains of B. oralis and B. ochraceus were sensitive to tetracycline, but 7 strains of B. melaninogenicus ss. melaninogenicus (including WAL 2721) were resistant to penicillin, and all strains of B. ochraceus were sensitive. The five strains of B. melaninogenicus ss. melaninogenicus and one of B. oralis (strain 7CM) were also resistant to methicillin. Two strains of B. ochraceus (10 and 79B) were resistant to methicillin, but sensitive to penicillin. Fifteen strains of B. melaninogenicus ss. melaninogenicus (including VPI 4196, VPI 7570A, WAL 2724, GUI 1011 and GUI 1034) and 4 strains of B. oralis (including NP333) were sensitive to colistin, but all strains of B. ochraceus were resistant.

G.L.C. analysis

All the test strains produced acetic acid. All strains of B. melaninogenicus ss. melaninogenicus and all except two strains of B. oralis produced moderate amounts (++) but only two strains of B. ochraceus (1956C and 2467B) produced this amount; the remaining two strains of B. oralis (VPI 5832 and 7CM) and 4 strains of B. ochraceus produced minor amounts (+) of acetic acid.

Twelve strains of B. melaninogenicus ss. melaninogenicus, 3 strains of B. oralis and 2 strains of B. ochraceus produced minor amounts of propionic acid; a further 8 strains of B. melaninogenicus ss. melaninogenicus, 4 strains of B. oralis and one strain of B. ochraceus produced trace amounts.

No strains of B. oralis or B. ochraceus produced iso-butyric acid, and only 4 strains of B. melaninogenicus ss. melaninogenicus produced trace amounts of this acid. No strain produced n-butyric acid.

Two strains of B. melaninogenicus ss. melaninogenicus (WAL 2721 and a laboratory isolate) produced minor amounts of iso-valeric acid; a further 16 strains of B. melaninogenicus ss. melaninogenicus and 4 strains of B. oralis but only one strain of B. ochraceus produced trace amounts of this acid. No strain produced n-valeric acid.

One laboratory isolate of B. melaninogenicus ss. melaninogenicus produced a moderate amount of lactic acid; 4 strains produced minor amounts and 16 strains produced trace amounts of this acid. Two strains of B. oralis produced minor amounts and two produced trace amounts of lactic acid. Strains of B. ochraceus, however, did not produce lactic acid except for a trace amount produced by strain 1956C.

All strains produced succinic acid; 14 strains of B. melaninogenicus ss. melaninogenicus, 6 strains of B. oralis and 3 strains of B. ochraceus produced moderate amounts and the remainder produced minor amounts of this acid.

The patterns of results indicate that differences between "-" and "trace" or "trace" and "+" results are probably less significant than differences between "-", "+" and "++" results.

TABLE 2

Percentage of specified results obtained with the three groups of test strains

Test	Result	% of test strains with the given result				
		B.mel. ss.mel. (23)	B.oralis (11)	B.ochraceus (6)	all non-pigmented strains (17)	all strains (40)
Growth in $O_2 + CO_2$	+	0	0	100	35.3	15
Pigment production	+	100	0	0	0	57.5
Tolerance tests:						
Taurocholate (T)	I	100	90.9	83.3	94.1	95
Deoxycholate (D)	I	100	100	100	100	100
T + D	I	100	100	100	100	100
Victoria Blue 4R	I	91.3	54.5	33.3	47.1	72.5
ethyl violet	I	100	90.9	100	94.1	97.5
gentian violet	I	100	100	100	100	100
brilliant green	I	100	100	100	100	100
Antibiotic disk resistance tests:						
neomycin (1000 ug)	S	95.7	90.9	100	94.1	95
kanamycin (1000 ug)	R	100	100	100	100	100
rifampicin	S	100	100	100	100	100
penicillin	S	78.3	90.9	100	94.1	85
methicillin	S	78.3	90.9	66.6	82.4	80
metronidazole	S	91.3	90.9	100	94.1	92.5
tetracycline	S	69.6	100	100	100	82.5
colistin	S	65.2	36.4	0	23.5	47.5
bacitracin	R	100	100	100	100	100
vancomycin	R	95.7	100	100	100	97.5
Biochemical tests:						
H_2S production	+	91.3	81.8	100	88.2	90
Haemolysis	+	100	81.8	0	52.9	80
Indole production	+	0	0	0	0	0
Aesculin hydrolysis	+	52.2	90.9	83.3	88.2	67.5
Dextran hydrolysis	+	65.2	45.5	100	64.7	65
Gelatin digestion	+	65.2	27.3	0	17.7	45
Lipase production	+	34.8	0	0	0	20

Test	Result	% of test strains with the given result				
		B.mel. ss.mel. (23)	B.oralis (11)	B.och-raceus (6)	all non-pigmented strains (17)	all strains (40)
Fermentation of:						
glucose	+	100	100	100	100	100
lactose	+	100	100	100	100	100
maltose	+	100	100	100	100	100
sucrose	+	100	100	100	100	100
rhamnose	+	4.3	63.6	0	41.2	20
trehalose	+	0	0	0	0	0
mannitol	+	0	0	0	0	0
G.L.C. analysis:						
acetic	+	0	18.2	66.7	35.3	15
"	++	100	81.8	33.3	64.7	85
propionic	-	13	36.4	50	41.2	25
"	tr	34.8	36.4	16.7	29.4	32.5
"	+	52.2	27.2	33.3	29.4	42.5
iso-butyric	-	82.6	100	100	100	90
"	tr	17.4	0	0	0	10
n-butyric	-	100	100	100	100	100
iso-valeric	-	21.7	63.6	83.3	70.6	42.5
"	tr	69.5	36.4	16.7	29.4	52.5
"	+	8.7	0	0	0	5
n-valeric	-	100	100	100	100	100
lactic	-	8.7	63.6	83.3	70.6	35
"	tr	69.6	18.2	16.7	17.6	47.5
"	+	17.4	18.2	0	11.8	15
"	++	4.3	0	0	0	2.5
succinic	+	39.1	45.5	50	47.1	42.5
"	++	60.9	54.5	50	52.9	57.5

COMMENTS

As a result of these studies with 40 test strains of B. melaninogenicus ss. melaninogenicus, B. oralis and B. ochraceus, the three groups were distinguished as follows:

B. melaninogenicus ss. melaninogenicus strains were strict anaerobes that produced brown, or occasionally black, colonies on media containing blood. They produced acid from glucose (and lactose, sucrose and maltose). They did not produce indole. In general, in disk tests, they were sensitive to neomycin (1000 µg disk), rifampicin and metronidazole, and they were resistant to kanamycin (1000 µg disk). They were inhibited in tolerance tests with two bile salts (separately and in combination), and they were inhibited by each of the four test dyes. Most strains were sensitive to penicillin. The results of tests for hydrolysis of aesculin and dextran, gelatin liquefaction, lipase production, and resistance to colistin varied between strains; there was no apparent relationship between the results of these tests.

In G.L.C. analysis, the test strains of this subspecies produced moderate amounts of acetic and succinic acids from glucose; propionic, iso-valeric and lactic acids were variable minor products.

B. oralis strains were strict anaerobes that failed to produce pigment, although the colonies of some strains on lysed blood agar became pale brown after incubation for 7 days. They were essentially similar to B. melaninogenicus ss. melaninogenicus strains and four laboratory isolates were indistinguishable from B. melaninogenicus ss. melaninogenicus except that they did not produce pigment. The four referred strains of B. oralis and three laboratory isolates were distinguished by the ability to produce acid from rhamnose and five of these strains were able to grow in the presence of Victoria blue 4R. The results of G.L.C. analysis were generally indistinguishable from the results obtained with B. melaninogenicus ss. melaninogenicus strains.

B. ochraceus strains were clearly distinguished by their ability to grow in air plus CO_2. They produced acid from glucose (and lactose, sucrose and maltose) but not from rhamnose, and did not produce indole. They were generally sensitive to disks of neomycin (1000 µg) and rifampicin, but resistant to metronidazole, kanamycin (1000 µg), and colistin. They hydrolysed aesculin (except one strain) and dextran. In G.L.C. analysis, they produced smaller amounts of acetic acid than the B. melaninogenicus ss. melaninogenicus and B. oralis groups; propionic acid was a variable minor product; they did not produce lactic, iso-butyric or iso-valeric acids.

The work reported here was financed by the Medical Research Council (MRC Grant No. G974/325B to Professor J.G. Collee). These results are conveyed to the members of the I.C.S.B. Taxonomic Sub-committee on Gram-negative anaerobic rods on the understanding that the Edinburgh team will submit a paper incorporating the data for publication in a scientific journal in the near future.

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APPENDIX IV

Publication from this thesis

Journal of Applied Bacteriology 1977, **42**, 259–273

The Classification of *Bacteroides melaninogenicus* and Related Species

W. P. HOLBROOK*, B. I. DUERDEN† AND A. G. DEACON

Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH8 9AG, Scotland

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One hundred and seventy-five strains of *Bacteroides melaninogenicus*, 17 strains of *B. oralis* and six strains of *B. ochraceus* were studied in a series of biochemical, chemical tolerance and antibiotic disc resistance tests and by the gas-liquid chromatographic analysis of the acid end products of metabolism. Strains of *B. melaninogenicus* ss. *asaccharolyticus* formed a distinct group with clear differences from other *B. melaninogenicus* strains. *B. melaninogenicus* ss. *intermedius* strains formed a homogeneous group that could be readily identified. *B. ochraceus* was distinguished from other *Bacteroides* spp. by its ability to grow in air enriched with CO_2. *Bacteriodes melaninogenicus* ss. *melaninogenicus* and *B. oralis* gave very similar patterns of results with the tests used and invariably were indistinguishable; the capacity to produce black-pigmented colonies on blood-containing media may not be a valid criterion for dividing these similar strains into two species.

THE SPECIES *Bacteroides melaninogenicus* contains all strictly anaerobic non-motile non-sporing Gram negative bacilli that produce black or brown pigmented colonies when grown on media containing blood. The pigment is an intracellular or cell-associated derivative of haemoglobin assimilated from the medium (Duerden 1975). This species was first described by Oliver & Wherry (1921) and the characteristic pigmentation remains the basic criterion for differentiation from other *Bacteroides* species (Holdeman & Moore 1974).

Bacteroides melaninogenicus strains are common commensals of the mouth (Socransky *et al.* 1963), lower gastro-intestinal tract (Drasar *et al.* 1969) and vagina (Gorbach *et al.* 1973) and have been implicated as significant pathogens in periodontal disease (MacDonald *et al.* 1960; Socransky 1970), bronchiectasis and lung abscess (Bartlett & Finegold 1972), post-operative wound infections (Finegold 1974) and uterine infections (Thadepalli, Gorbach & Keith 1973). The species, however, is a heterogeneous group. Moore & Holdeman (1973) recognize three subspecies of *B. melaninogenicus*: ss. *melaninogenicus*, ss. *intermedius* and ss. *asaccharolyticus*. These subspecies have a number of distinguishing characteristics but can be simply identified on the basis of glucose fermentation and the production of indole (Duerden *et al.* 1976).

Non-pigmented strains of Gram negative anaerobic bacilli were isolated from the human mouth and designated *B. oralis* by Loesche *et al.* (1964); in other respects, these strains are very similar to some strains of *B. melaninogenicus* ss. *melaninogenicus*. The

* Present address: Department of Oral Medicine, Turner Dental School, University of Manchester, Bridgeford Street, Manchester M15 6FH, England.
† Present address: Department of Medical Microbiology, Sheffield University Medical School, Beech Hill Road, Sheffield S10 2RX, England.

similarities are such that one reference strain described as *B. oralis* was later found to produce pigment and has to be regarded as *B. melaninogenicus* (Holbrook & Duerden 1974). Other seemingly related strains were initially designated *B. oralis* var. *elongatus* (Loesche *et al.* 1964); they require carbon dioxide for growth but are not strict anaerobes and are now called *B. ochraceus* (Holdeman & Moore 1972). The present study was undertaken to investigate the characteristics and interrelationships of the three subspecies of *B. melaninogenicus*, and of *B. oralis* and *B. ochraceus*.

Materials and Methods

Organisms

Bacteroides melaninogenicus

A total of 175 strains was studied. Strains 9336, 9337 and 9338 were obtained from the National Collection of Type Cultures (NCTC), Central Public Health Laboratory, Colindale Avenue, London NW9 5HT. Strain 15930 (formerly *B. oralis*; see Holbrook & Duerden 1974) was from the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 28052, U.S.A. Strains WAL 2721 and WAL 2724 were from Dr S. M. Finegold, Wadsworth General Hospital, Veterans Administration, Los Angeles, California, 90024, U.S.A. Strains GUI 1011 and GUI 1034 were from Dr K. Ueno, Department of Bacteriology, Gifu University Medical School, Tsukasa-Machi, Gifu-shi, Gifu-ken, Japan. Strain VPI 4196 was from Dr E. Cato, Virginia Polytechnic Institute and State University, Blacksburg, Va. 24060, U.S.A. Five strains were from Professor G. Sundqvist, Department of Endodontics, University of Umeå, Sweden. Three strains were isolated from abdominal wound infections by Dr R. Wiseman, Bangour General Hospital, W. Lothian, Scotland. Two strains were from Dr J. M. Hardie, London Hospital Medical College, Dental School, Turner Street, London E1 2AD. Four strains were isolated from wound swabs and one from an intravenous catheter in the Bacteriology Laboratory, Royal Infirmary, Edinburgh. Seven strains were isolated in our laboratory from oral infections (two from dental abscesses, two from wound swabs following oral surgery, and one each from cases of Vincent's infection, pericoronitis and a root canal infection). Ninety-four strains were isolated from subgingival dental plaque in our laboratory. Thirty-one strains were isolated from high vaginal swabs (19 by Dr B. Watt, Central Microbiological Laboratories, Western General Hospital, Edinburgh, and 12 in the Bacteriology Laboratory, Royal Infirmary, Edinburgh). Nineteen strains were isolated in our laboratory from specimens of faeces sent to the Bacteriology Laboratory, Royal Infirmary, Edinburgh.

Bacteroides oralis

Seventeen strains were studied. Strains J1, 7CM and 30 were from Dr B. A. Phillips, National Institute for Research in Dairying, University of Reading, England. Strains VPI 5832 and VPI 7570A were from Dr W. E. C. Moore, Virginia Polytechnic Institute. Strain NP 333 was from Mr G. H. Bowden, London Hospital Medical College. Eleven strains were isolated in our laboratory from subgingival dental plaque.

Bacteroides ochraceus

Six strains were studied. Strain VPI 2845 was from Dr E. Cato. Strains 1956C and 2467B were from Dr M. Sebald, Institut Pasteur, 25 Rue du Docteur Roux, Paris. Strains 10, 79B and 73 were from Dr W. H. van Palenstein-Helderman, Preventive Dentistry Department, University of Utrecht, Netherlands.

Forty-one strains of *B. melaninogenicus* and one strain each of *B. oralis* and *B. ochraceus* were included in previous studies on the characterization of clinically-important Gram negative anaerobic bacilli (Duerden *et al.* 1976). *Bacteroides melaninogenicus* strains WAL 2721, WAL 2724, GUI 1011, GUI 1034 and ATCC 15930, *B. oralis* strains VPI 5832, VPI 7570A, J1, 7CM and 30, and *B. ochraceus* strains 1956C and 2467B were studied as part of the collaborative study instigated by the International Committee for Systematic Bacteriology (ICSB) Taxonomic Sub-Committee on Gram negative anaerobic rods.

Characterization of strains

Ninety-two strains of *B. melaninogenicus*, 15 strains of *B. oralis* and the six strains of *B. ochraceus* were subjected to the following series of morphological, biochemical, chemical tolerance and antibiotic disc resistance tests (for details of media and methods, see Duerden *et al.* 1976).

Morphological and biochemical tests

Microscopic and colonial morphology; haemolytic effect on blood agar; pigment production; motility; lipase activity; oxidase test; catalase test; hydrogen sulphide production; indole production; gelatinase test; aesculin hydrolysis; fermentation of glucose, lactose, sucrose, maltose, rhamnose, trehalose and mannitol.

Chemical tolerance tests

Growth in the presence of (1) the bile salts sodium taurocholate, sodium deoxycholate, separately and in combination, and (2) the dyes brilliant green, Victoria blue 4R, gentian violet and ethyl violet (separately).

Antibiotic disc resistance tests

Resistance to discs containing neomycin 1000 μg and 10 μg, kanamycin 1000 μg and 30 μg, penicillin 1·5 units, methicillin 10 μg, erythromycin 60 μg, colistin 10 μg, rifampicin 15 μg, lincomycin 2 μg, clindamycin 2 μg, bacitracin 0·1 unit, vancomycin 15 μg, chloramphenicol 10 μg, tetracycline 10 μg, and metronidazole 5 μg.

The remaining 85 strains were identified by the following short, combined scheme derived from the above series of tests: pigment production; antibiotic disc resistance tests with neomycin 1000 μg, kanamycin 1000 μg, penicillin 1·5 units, and rifampicin 15 μg discs; separate tolerance tests with sodium taurocholate, sodium deoxycholate, Victoria blue 4R and ethyl violet; biochemical tests for the production of indole,

digestion of gelatin, and hydrolysis of aesculin; and the fermentation of glucose, rhamnose, trehalose and mannitol.

GLC analysis of short-chain fatty acids

One hundred and two strains of *B. melaninogenicus,* 13 strains of *B. oralis* and six strains of *B. ochraceus* were subjected to gas-liquid chromatographic (GLC) analysis of their short-chain fatty acid products of metabolism.

Medium

The strains were grown in two tubes of a proteose peptone medium (PPYS) containing (w/v): proteose peptone (Oxoid) 2%; yeast extract (Difco) 1%; and NaCl 0·5%. Filter-sterilized solutions of the following heat-labile supplements were added aseptically to the (cooled) autoclaved basal medium (pH 7·4) to give final concentrations of: inactivated horse serum, 2%; haemin, 5 μg/ml; menadione, 1 μg/ml; cysteine hydrochloride, 0·075%; and Na_2CO_3, 0·04%. Glucose, 1%, was added to one tube (PPYSG medium). The pH value at inoculation was $7·1 \pm 0·1$.

The inoculum was one drop (0·02 ml) of a 48 h culture in cooked-meat broth. After anaerobic incubation (see Collee *et al.* 1972) for 48 h, or longer for slow-growing strains, the cultures were examined microscopically for growth and the pH value of each tube was measured for comparison with the results of the glucose fermentation tests (above). Thirteen strains were tested after growth in PPYS medium without added glucose.

Chromatograph

A Pye-Unicam series 104 gas chromatograph fitted with heated injection ports and dual flame-ionization detectors was operated isothermally at 190 °C with a detector temperature of 250 °C. The carrier-gas was oxygen-free nitrogen at a flow-rate of 35 ml/min and the hydrogen flow-rate of both detectors was adjusted for optimum sensitivity. The instrument was fitted with two identical glass columns (1·5 m × 4 mm) containing Chromosorb 101 (Johns-Manville Corp., U.S.A.; supplied by Gas Chromatography Services Ltd., 23 Old Chester Road, Lower Bebington, Wirral, Merseyside L63 7LA). Columns were packed in the laboratory. The recorder was a Servoscribe 1S model 541.20 (Belmont Instruments, 6 Belmont Drive, Giffnock, Glasgow G46 7PA) set at the 10 mV range with a recorder speed of 120 mm/h.

Analysis

The procedures used were derived from those of Carlsson (1973).

(1) *Volatile acids.* Cultures were acidified with 50% sulphuric acid to pH 2·0 and a 0·6 μl sample of cell-free supernatant was injected directly on to the analysing column without further pre-treatment or extraction. Contamination of the top 2–3 cm of the column occurred during use and required periodic replacement with fresh polymer. The attenuation setting was 2×10^{-2} at the ×1 range setting.

(2) *Non-volatile acids.* The acidified culture supernates were methylated according to the method of Holdeman & Moore (1972) and a 0·6 μl sample of the chloroform ex-

tract was injected on to the column under the same conditions as those used for the volatile acids but with an attenuation setting of 5×10^{-2}. Lactic and succinic acids were detected qualitatively in the analysis of volatile acids, but this was confirmed quantitatively by the methylation procedure.

Standards

Single and combined 0·01 M aqueous standards of the volatile acids were used to establish absolute and relative retention times; 0·02 M standards were used in the analysis of the non-volatile acids. Samples of uninoculated (sterile) PPYSG medium were included as controls in every batch of each of the two types of analysis. A PPYSG control and a combined acid standard were used to monitor retention times and the sensitivity settings of the instrument as a routine each day. The approximate concentration values of acids for test samples were calculated by comparing the corrected peak heights of the test samples with those of the appropriate acid standards. Results were recorded as follows:

Volatile acids. + +, Concentration value >10 μmol/ml; +, 1·1–10 μmol/ml; tr (trace), 0·2–1·0 μmol/ml; –, <0·2 μmol/ml.

Lactic and succinic acids. + +, Concentration value >20 μmol/ml; +, 10–20 μmol/ml; tr, 1–9 μmol/ml; –, <1 μmol/ml.

Results

The test strains were provisionally allocated to one of three species:

(1) Strictly anaerobic strains that produced black or brown pigmented colonies when grown on lysed-human-blood agar for up to one week were assigned to the species *B. melaninogenicus*. The three subspecies of *B. melaninogenicus* were distinguished by tests for the production of indole and the fermentation of glucose:

(a) Strains that did not produce acid from glucose were labelled ss. *asaccharolyticus* (46 strains). These included strain NCTC 9337.

(b) Strains that produced acid from glucose and produced indole were labelled ss. *intermedius* (78 strains). These included strains NCTC 9336 and 9338.

(c) Strains that produced acid from glucose but did not produce indole were labelled ss. *melaninogenicus* (53 strains). These included strains ATCC 15930, WAL 2721 and 2724, GUI 1011 and 1034, VPI 4196 and 7570A and 30.

(2) Strictly anaerobic, non-pigmented strains that were inhibited by bile salts were assigned to the species *B. oralis* (15 strains). Two strains (VPI 7570A and 30) were submitted to us as strains of *B. oralis* but produced black or brown pigmented colonies and were transferred to our *B. melaninogenicus* ss. *melaninogenicus* group (above).

(3) Non-pigmented strains that were able to grow in 10% CO_2 in air were assigned to the species *B. ochraceus* (6 strains).

Cell morphology

All strains were Gram negative bacilli or cocco-bacilli and many were pleomorphic. *B. melaninogenicus* and *B. oralis* strains were predominantly cocco-bacilli or short bacilli with rounded ends, often arranged in short chains. A few longer rods were seen and some strains were highly pleomorphic. *B. ochraceus* strains were long, slender bacilli with rounded or tapered ends and often with a central oval swelling.

Colony morphology

The colonies of *B. melaninogenicus* ss. *asaccharolyticus* were 0·5 mm diameter, round, convex and opaque. Individual colonies were light grey after incubation for 48 h but confluent growth was sometimes brown and appeared moist; colonies were 1 mm in diameter, dark brown or black after further incubation on lysed-blood agar. After one week, some strains produced very small variant colonies that were light brown in colour.

Colonies of *B. melaninogenicus* ss. *intermedius* were 1–2 mm in diameter, round, convex and opaque. After incubation for 48 h individual colonies were grey but confluent growth was becoming black; all colonies were black after further incubation.

Colonies of *B. melaninogenicus* ss. *melaninogenicus* were 1–2 mm in diameter, round, convex and opaque. After incubation for 48 h they were typically light grey, becoming brown after further incubation. The pigmentation varied between strains from light brown to almost black. The colonies of many strains had a light brown annulus around a dark brown centre.

All strains of *B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius* produced zones of complete or incomplete haemolysis on human-blood agar; the development of these zones paralleled the development of pigment. Many strains of *B. melaninogenicus* ss. *asaccharolyticus* were similarly haemolytic but some strains of this subspecies were non-haemolytic and, therefore, non-pigmented on human-blood agar although all of these strains produced black pigment when grown on lysed-blood agar.

Colonies of *B. oralis* were 1–2 mm in diameter, round, convex, opaque and grey; they tended to coalesce. After incubation for 7 d, the colonies of some strains (including VPI 5832 and NP 333) became light brown and were difficult to distinguish from the lighter-pigmented strains of *B. melaninogenicus* ss. *melaninogenicus*.

Bacteroides ochraceus strains typically produced two colony types: (a) 1 mm diameter, round or with an irregular edge, smooth, opaque and blue-grey; (b) 1 mm in diameter, rhizoid, granular and ochre in colour.

Biochemical tests

None of the strains tested produced oxidase or catalase; all strains produced hydrogen sulphide although some strains produced only small amounts. The results of the other biochemical tests are shown in Table 1. Strains of *B. melaninogenicus* ss. *asaccharolyticus* (46) did not ferment any of the test carbohydrates and did not hydrolyse aesculin or dextran; most strains produced indole and digested gelatin; only a few strains produced lipase. All strains of *B. melaninogenicus* ss. *intermedius* (78) fermented glucose, sucrose and maltose, but not rhamnose; a few strains fermented lactose but only two strains fermented mannitol and one strain fermented trehalose. All strains pro-

duced indole and only one strain failed to digest gelatin; only three strains hydrolysed dextran and one strain hydrolysed aesculin. Many strains produced lipase. *Bacteroides melaninogenicus* ss. *melaninogenicus* strains (53) fermented glucose but not trehalose or mannitol; only one strain failed to ferment maltose, another failed to ferment sucrose, and two strains failed to ferment lactose. Two strains fermented rhamnose. The strains assigned to this subspecies did not produce indole. In tests for the hydrolysis of aesculin and dextran and the digestion of gelatin this subspecies did not give a constant pattern; some strains gave negative results in all three tests whereas other strains gave positive results in one, two or all three tests. There was no correlation between the results obtained in these three separate tests. A few strains produced lipase.

Bacteroides oralis strains (15) fermented glucose, lactose, sucrose and maltose, but not trehalose or mannitol; eight strains fermented rhamnose. All except two strains hydrolysed aesculin; some strains hydrolysed dextran and some strains digested gelatin. None of the strains produced indole or lipase.

Bacteroides ochraceus strains (6) fermented glucose, lactose, sucrose and maltose, but not rhamnose, trehalose or mannitol. All strains hydrolysed dextran and only one strain failed to hydrolyse aesculin. None of the strains digested gelatin or produced indole or lipase.

Chemical tolerance tests

The results of these tests are shown in Table 2. All the test strains of *B. melaninogenicus* and *B. ochraceus* and all except one strain of *B. oralis* (7CM) were inhibited by bile salts. All strains were inhibited by ethyl violet, gentian violet and brilliant green except one strain of *B. oralis* (7CM) that grew in the presence of ethyl violet and one strain each of *B. melaninogenicus* ss. *melaninogenicus* and ss. *asaccharolyticus* that grew in the presence of brilliant green. The atypical strain of *B. oralis* (7CM) gave a pattern of results in biochemical, chemical tolerance and antibiotic disc resistance tests that was more typical of a strain of the *B. fragilis* group.

Antibiotic disc resistance tests

The results of these tests are shown in Table 3. All strains of the anaerobic species were sensitive to metronidazole and one strain of *B. ochraceus* was also sensitive to this agent. All except one strain of *B. melaninogenicus* ss. *intermedius* and two strains of ss. *melaninogenicus* were resistant to kanamycin (1000 μg disc). Most strains were sensitive to neomycin, but 19 strains of *B. melaninogenicus* ss. *asaccharolyticus,* five strains of ss. *intermedius,* three strains of ss. *melaninogenicus* and one strain of *B. oralis* were resistant. Most strains were sensitive to penicillin, but four strains of *B. melaninogenicus* ss. *asaccharolyticus*, six strains of ss. *intermedius*, 14 strains of ss. *melaninogenicus*, and two strains of *B. oralis* were resistant. The results obtained with methicillin and penicillin discs were the same for strains tested with both discs, except that two strains of *B. ochraceus* were sensitive to penicillin but resistant to methicillin. Almost all strains of *B. ochraceus, B. oralis, B. melaninogenicus* ss. *melaninogenicus* and ss. *intermedius* were resistant to the vancomycin disc but most strains of ss. *asaccharolyticus* were sensitive.

TABLE 1

Results of biochemical tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth in air + CO_2	0/46	0/78	0/53	0/15	6/6
Pigment production (black/brown)	46/46	78/78	53/53	0/15	0/6
Indole production	42/46	78/78	0/53	0/15	0/6
Aesculin hydrolysis	0/46	1/78	17/53	14/15	5/6
Gelatin digestion	45/46	77/78	30/53	7/15	0/6
Dextran hydrolysis	0/44†	3/75‡	28/53	5/15	6/6
Lipase production	5/22*	31/39	9/26	0/13	0/6
Fermentation of:					
glucose	0/46	78/78	53/53	15/15	6/6
lactose	0/29	8/39	24/26	13/13	6/6
sucrose	0/29	39/39	25/26	13/13	6/6
maltose	0/29	39/39	25/26	13/13	6/6
rhamnose	0/46	0/78	2/53	8/15	0/6
trehalose	0/46	1/78	0/53	0/15	0/6
mannitol	0/46	2/78	0/53	0/15	0/6

* Seven strains failed to grow on test medium.
† Two strains failed to grow on test medium.
‡ Three strains failed to grown on test medium.

TABLE 2
Results of tolerance tests with 198 test strains

Test result	Number of positive strains/number tested in each group				
	B. melaninogenicus				
	ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Growth on basal medium	44/46	75/78	53/53	14/15	6/6
Growth on basal medium plus:					
taurocholate (0·5%)	0/44	0/75	0/53	1/14	0/6
deoxycholate (0·1%)	0/44	0/75	0/53	1/14	0/6
taurocholate (0·5%) and deoxycholate (0·1%)	0/28	0/39	0/26	1/13	0/6
Victoria blue 4R (1/80 000)	7/44	4/75	6/53	7/14	4/6
ethyl violet (1/80 000)	0/44	0/75	0/53	1/14	0/6
gentian violet (1/100 000)	0/28	0/39	0/26	0/13	0/6
brilliant green (1/80 000)	1/28	0/39	1/25	0/13	0/6

TABLE 3

Results of antibiotic disc resistance tests with 198 test strains

Test result		Number of positive strains/number tested in each group				
		B. melaninogenicus				
		ss. *asaccharolyticus*	ss. *intermedius*	ss. *melaninogenicus*	*B. oralis*	*B. ochraceus*
Neomycin (1000 μg)	S	27/46	73/78	50/53	14/15	6/6
Kanamycin (1000 μg)	R	46/46	77/78	51/53	15/15	6/6
Penicillin (1·5 unit)	S	42/46	72/78	39/53	13/15	6/6
Methicillin (10 μg)	S	25/29	37/39	20/26	11/13	4/6
Erythromycin (60 μg)	S	29/29	39/39	26/26	13/13	6/6
Tetracycline (10 μg)	S	24/29	35/39	24/26	12/13	6/6
Colistin (10 μg)	S	12/29	38/39	18/26	4/13	0/6
Rifampicin (15 μg)	S	46/46	78/78	51/53	15/15	6/6
Lincomycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Clindamycin (2 μg)	S	29/29	39/39	25/26	13/13	6/6
Bacitracin (0·1 unit)	R	4/29	17/39	25/26	12/13	6/6
Vancomycin (15 μg)	R	9/29	38/39	24/26	13/13	5/6
Chloramphenicol (10 μg)	S	28/29	39/39	26/26	13/13	6/6
Metronidazole (5 μg)	S	29/29	39/39	26/26	13/13	1/6

R, resistant; S, sensitive.

GLC analysis

The results of these tests are shown in Table 4. All strains produced acetic acid. Strains of *B. melaninogenicus* ss. *asaccharolyticus* produced *n*-butyric acid and lactic acid as major products; nine strains did not produce succinic acid but seven strains produced significant amounts of this acid. The two strains that produced >10 μmol/ml of succinate had been incubated for 7 d to obtain good growth; they also produced larger quantities of acetic, *n*-butyric and lactic acids. All strains of ss. *asaccharolyticus* produced significant amounts of propionic acid and smaller amounts of iso-butyric and iso-valeric acids. In general, the saccharolytic organisms i.e. *B. melaninogenicus* ss. *intermedius* and ss. *melaninogenicus, B. oralis* and *B. ochraceus*, produced succinic acid as a major product but did not produce *n*-butyric acid. However, three strains assigned to the *B. melaninogenicus* ss. *intermedius* group consistently produced *n*-butyric acid but not succinic acid in repeated tests. Strains of *B. melaninogenicus* ss. *intermedius* and ss. *melaninogenicus* produced variable amounts of lactic acid; propionic, iso-butyric, and iso-valeric acids were minor products of most strains. None of the test strains of *B. oralis* produced iso-butyric acid; propionic, iso-valeric and lactic acids were minor products of some strains. In general, the test strains of *B. ochraceus* produced significant amounts of acetic and succinic acids only, but they produced smaller amounts of acetic acid than the other test strains. None of the test strains of *B. ochraceus* produced iso-butyric acid but three strains produced propionic acid and one strain produced iso-valeric and lactic acids as minor products.

Identification of strains from different sites

Strains of *B. melaninogenicus* were isolated from the mouth, the vagina, and from faeces; almost all the oral strains were identified as ss. *melaninogenicus* or ss. *intermedius* whereas all three subspecies were regularly isolated from the vagina and from faeces. All the strains of *B. oralis* and *B. ochraceus* were isolated from the mouth except for one strain of *B. oralis* that was isolated from a putrid lung abscess.

Discussion

Moore & Holdeman (1973) divided the pigmented bacteroides into three subspecies: *B. melaninogenicus* ss. *melaninogenicus,* ss. *intermedius* and ss. *asaccharolyticus*. The studies of Williams *et al.* (1975) on cell wall composition and DNA base ratios supported this classification. Lambe (1974) and Lambe & Jerris (1976) have distinguished between the same three groups of fluorescent antibody staining and have also subdivided the ss. *intermedius* strains into two sero-groups. Werner *et al.* (1971), however, would not include saccharolytic and asaccharolytic organisms in the same species. They recognized only the asaccharolytic strains as *B. melaninogenicus* but they did not distinguish the saccharolytic strains from non-pigmented bacteroides. The results of the present study confirm the separation of the pigmented bacteroides strains into three broad groups.

Strains of *B. melaninogenicus* ss. *asaccharolyticus* are clearly distinguished by the failure to ferment carbohydrates and the production of significant amounts of *n*-butyric

TABLE 4

Results of GLC analysis of acid end-products from 121 test strains

Acid concentration		Number of strains in each group*				
		B. melaninogenicus				
		ss. *asaccharolyticus* (18†)	ss. *intermedius* (36†)	ss. *melaninogenicus* (48†)	*B. oralis* (13)	*B. ochraceus* (6)
Acetic	++	13	31	44	9	2
	+	5	5	4	4	4
Propionic	++	5	1			
	+	13	16	19	4	2
	tr		11	21	5	1
	−		8	8	4	3
iso-Butyric	+	16	4			
	tr	2	27	16		
	−		5	32	13	6
n-Butyric	++	7				
	+	11	2			
	tr		1			
	−		33	48	13	6
iso-Valeric	++	1				
	+	17	18	11		
	tr		18	27	5	1
	−			10	8	5
n-Valeric	−	18	36	48	13	6
Lactic	++	17	19	11		
	+	1	11	14	3	
	tr		6	21	2	1
	−			2	8	5
Succinic	++	2	32	38	7	3
	+	5	1	10	6	3
	tr	2	1			
	−	9	2			

* Numbers in parentheses indicate number of strains tested in each group.

† Seven strains of *B. melaninogenicus* ss. *intermedius*, five strains of ss. *melaninogenicus* and one strain of ss. *asaccharolyticus* were tested in medium (without added glucose) only.

acid; they produce indole and are proteolytic. Werner *et al.* (1971) and Williams *et al.* (1975) did not detect the production of succinic acid by any of their test strains of the asaccharolytic group. The production of minor quantities of succinate by seven asaccharolytic strains under the conditions of our tests is consistent with the findings of Holdeman & Moore (1972). The ICSB Taxonomic Sub-committee on Gram negative anaerobic rods has recently clarified the taxonomic status of the asaccharolytic strains and has suggested that *B. melaninogenicus* ss. *asaccharolyticus* is sufficiently different from the other subspecies to be regarded as a separate species designated *B. asaccharolyticus* (Anon. 1977, in press).

The saccharolytic strains of *B. melaninogenicus* could be divided into two clear groups on the basis of our series of tests. The strains that produced indole appeared to be a fairly homogeneous group that corresponds with the subspecies *intermedius* (Holdeman & Moore 1974). Strains that failed to produce indole were designated *B. melaninogenicus* ss. *melaninogenicus*; many strains were easily separated from strains of ss. *intermedius* by their distinctive colony morphology (see Results) that has also been observed by Lambe & Jerris (1976). GLC analysis of the acid end-products of metabolism was not helpful in differentiating between individual strains of these two subspecies. There were differences between the median concentrations of acid end-products but there was considerable overlapping between the ranges of concentrations obtained with the two subspecies. *B. melaninogenicus* ss. *melaninogenicus* did not form a homogeneous group but defined sub-groups could not be detected on the basis of the results of biochemical, chemical tolerance or antibiotic disc resistance tests.

Some of the non-pigmented strains of bacteroides that were identified as *B. oralis* closely resembled strains of *B. melaninogenicus* ss. *melaninogenicus* in the series of tests adopted in this study. They were differentiated only by their failure to produce pigment; this distinction was even less clear with strains of *B. melaninogenicus* ss. *melaninogenicus* that produced brown pigmented colonies only slowly and strains of *B. oralis* that gave buff-coloured colonies after prolonged incubation. However, some strains identified as *B. oralis* grew in the presence of Victoria blue 4R and fermented rhamnose; these strains were more clearly distinguished from *B. melaninogenicus* ss. *melaninogenicus*. As a result of the similarities between some strains of *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis*, the relationship between these two groups has been the subject of some taxonomic debate that remains unresolved (Anon. 1977, in press). Terada *et al.* (1976) have recently suggested that *B. oralis* and *B. ruminicola* might be closely related groups that have many similarities with *B. melaninogenicus* ss. *melaninogenicus*. In a numerical taxonomic study, Sundqvist (1976) found close similarities between strains of *B. melaninogenicus* ss. *melaninogenicus*, *B. oralis* and *B. ruminicola*; he did not regard pigment production as a good basis for differentiation between *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis*. It remains to be decided whether the ability to assimilate haemoglobin and produce brown pigmented colonies when grown on media containing blood is a valid criterion for dividing these very similar strains of *B. melaninogenicus* ss. *melaninogenicus* and *B. oralis* into two species.

B. ochraceus strains were clearly differentiated from the other test strains; they were able to grow in air plus CO_2 and were resistant to metronidazole (except for one aberrant strain), an antimicrobial agent to which only anaerobic bacteria are susceptible (Prince *et al.* 1969). The status of these strains within the genus *Bacteroides* requires

further investigation but on the present evidence it would seem that they should be removed from the genus.

Holdeman & Moore (1974) stressed the value of GLC analysis in the identification of Gram negative anaerobic bacilli. In our studies GLC analysis distinguished between asaccharolytic and saccharolytic strains although three strains that gave positive results in fermentation tests with glucose, and were therefore identified as *B. melaninogenicus* ss. *intermedius,* gave GLC patterns typical of ss. *asaccharolyticus*. These strains produced *iso*-butyric and *iso*-valeric acids and are, therefore, not fusobacteria. They require further investigation to determine whether they constitute a new sub-group or species. We were unable to distinguish between individual strains of *B. melaninogenicus* ss. *intermedius* and strains of the *B. melaninogenicus* ss. *melaninogenicus/B. oralis* group.

Most of our test strains were readily identified from the pattern of results obtained in the short, combined set of biochemical, chemical tolerance and antibiotic disc resistance tests. The results in chemical tolerance and antibiotic disc resistance tests distinguish the groups of bacteroides studied in this investigation from other Gram negative anaerobic bacilli. These tests are used as a combined set to prevent an anomolous result in any single test leading to an incorrect identification. The results of the biochemical tests provide the basis for the differentiation between the species and subspecies within this group of bacteroides.

We thank Professor J. G. Collee for much helpful advice and encouragement and Mr R. Brown and his colleagues for their skilled technical assistance. We thank the many colleagues who donated strains for this study and we are indebted to Professor J. Boyes of the Edinburgh Dental School for facilities for taking gingival samples. W. P. Holbrook thanks the University of Edinburgh for a Faculty of Medicine Scholarship. Financial support from the Medical Research Council (MRC Grant No. G 974/325 B) is gratefully acknowledged.

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APPENDIX V

Title page and summary of a paper accepted for publication in the Journal of Medical Microbiology. This paper incorporated some of the results presented in this thesis. The letter of acceptance from the editor is enclosed. Prospective date of publication

J. med. Microbiol, (1978) 11 (1) February 1978.

GAS LIQUID CHROMATOGRAPHIC ANALYSIS OF METABOLIC PRODUCTS IN THE IDENTIFICATION OF BACTEROIDACEAE OF CLINICAL INTEREST

A.G. DEACON, B.I. DUERDEN* & W.P. HOLBROCK

Department of Bacteriology, Edinburgh University Medical School, Teviot Place, EDINBURGH, EH8 9AG.

Running title: Identification of Bacteroidaceae by GLC

Proofs to be sent to: A.G. DEACON,
Department of Bacteriology,
Edinburgh University Medical School,
Teviot Place,
EDINBURGH, EH8 9AG.

* Present address: Department of Medical Microbiology,
Sheffield University Medical School,
Beech Hill Road,
SHEFFIELD, S10 2RX.

Present address: Department of Oral Medicine,
Turner Dental School,
University of Manchester,
Bridgeford Street,
MANCHESTER, M15 6FH

SUMMARY

The acid end-products of 188 isolates from the family Bacteroidaceae were separated and analysed by gas-liquid chromatography on broth cultures. A range of media were evaluated and definitive studies were performed in a fully supplemented complex medium. The limitations of this approach to the identification of a wide range of strains from various clinical sources were determined and the results were correlated with those of a series of morphological, biochemical, tolerance and antibiotic resistance tests.

All test strains were identified to generic level on the basis of simple microscopic and colonial observations and GLC analysis; additional tests were required to allow species or subspecies identification of most strains. Population differences were detected between some species or subspecies by quantitative analyses of fatty acids, but individual strains could not always be separated because of overlapping ranges of distribution of acids that were common products of more than one species or subspecies. Small differences in minor products between different species or subspecies were variable and are not considered adequate for discrimination at these taxonomic levels without support from other observations.

The potential application of the GLC technique to the rapid and accurate identification of these organisms in hospital laboratories is considered.

UNIVERSITY OF DUBLIN

Professor J. P. Arbuthnott

DEPARTMENT OF MICROBIOLOGY
MOYNE INSTITUTE
TRINITY COLLEGE
DUBLIN 2
IRELAND

Tel. 772941
Ext. 390

2nd June, 1977

Dr. A.G. Deacon,
Department of Bacteriology,
University Medical School,
Teviot Place,
Edinburgh EG8 9AG.
SCOTLAND.

Dear Dr. Deacon,

Deacon, A.G., Duerden, B.I. and Holbrook, W.P. "Gas liquid chromatographic analysis of metabolic products in the identification of bacteroidaceae of clinical interest

Your manuscript has been forwarded to me as a member of the Editorial Board. I have read it with great interest and consider that you and your colleagues have made a substantial contribution to an important area of Medical Microbiology. I am writing to inform you that I intend to recommend your manuscript for publication in the Journal. At the same time I must apologise that I have not yet been able to complete the editorial work. I undertake to do this as soon as possible and in the meantime I may be in touch with you to clarify some points of detail.

Best wishes.

Yours sincerely,

John Arbuthnott

J.P. Arbuthnott, Ph.D.
Professor of Microbiology.

TABLE 3

Results obtained with I.C.S.B. referred strains

Test	WAL 2721	WAL 2724	GUI 1011	GUI 1034	ATCC 15930	VPI 30	VPI 7570A	VPI 5832	J1	7CM	1956C	2467B
Growth in air + CO_2	−	−	−	−	−	−	−	−	−	−	+	+
Pigment production	+	+	+	+	+	+	+	±†	−	−	−	−
Tolerance tests:												
Taurocholate (T)	I	I	I	I	I	I	I	I	I	+	I	I
deoxycholate (D)	I	I	I	I	I	I	I	I	I	+	I	I
T + D	I	I	I	I	I	I	I	I	I	+	I	I
Victoria blue 4R	I	+	I	I	I	I	I	I	+	+	I	+
ethyl violet	I	I	I	I	I	I	I	I	I	+	I	I
gentian violet	I	I	I	I	I	I	I	I	I	I	I	I
brilliant green	I	I	I	I	I	I	I	I	I	I	I	I
Indole production	−	−	−	−	−	−	−	−	−	−	−	−
Fermentation of:												
glucose	+	+	+	+	+	+	+	+	+	+	+	+
lactose	+	+	+	+	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+	+	+	+	+
maltose	+	+	+	+	+	+	+	+	+	+	+	+
trehalose	−	−	−	−	−	−	−	−	−	−	−	−
mannitol	−	−	−	−	−	−	−	−	−	−	−	−
rhamnose	−	−	−	−	−	−	−	+	+	+	−	−
Aesculin hydrolysis	−	−	+	+	+	+	+	+	+	−	−	+
Dextran hydrolysis	−	+	−	−	+	+	−	−	−	−	+	+
Gelatin digestion	−	−	+	+	+	−	+	−	−	−	−	−
Lipase production	−	−	−	−	+	+	−	−	−	−	−	−
Haemolysis	+	+	+	+	+	+	+	+	+	+	−	−
H_2S production	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase production	−	−	−	−	−	−	−	−	−	−	−	−
Catalase production	−	−	−	−	−	−	−	−	−	−	−	−
Antibiotic tests:												
neomycin 1000 µg	S	S	S	S	S	R	S	S	S	R	S	S
neomycin 10 µg	R	R	R	R	R	R	R	R	R	R	R	R
kanamycin 1000 µg	R	R	R	R	R	R	R	R	R	R	R	R
kanamycin 30 µg	R	R	R	R	R	R	R	R	R	R	R	R
penicillin 1·5 units	R	S	S	S	S	S	S	S	S	R	S	S
methicillin 10 µg	R	S	S	S	S	S	S	S	S	R	S	S
erythromycin 60 µg	S	S	S	S	S	S	S	S	S	S	S	S
tetracycline 10 µg	R	S	S	S	S	S	S	S	S	S	S	S
colistin 10 µg	R	S	S	S	R	R	S	R	R	R	R	R
rifampicin 15 µg	S	S	S	S	S	S	S	S	S	S	S	S
lincomycin 2 µg	S	S	S	S	S	S	S	S	S	S	S	S
clindamycin 2 µg	S	S	S	S	S	S	S	S	S	S	S	S
bacitracin 0·1 unit	R	R	R	R	R	R	R	R	R	R	R	R
vancomycin 15 µg	R	R	R	R	S	R	R	R	R	R	S	R
chloramphenicol 10 µg	S	S	S	S	S	S	S	S	S	S	S	S
metronidazole 5 µg	S	S	S	S	R	R	S	S	S	S	R	R
G.L.C. analysis												
acetic	++	++	++	++	++	++	++	+	++	+	++	++
propionic	+	+	+	G	G	G	+	−	G	G	G	+
iso-butyric	G	−	−	−	−	−	−	−	−	−	−	−
n-butyric	−	−	−	−	−	−	−	−	−	−	−	−
iso-valeric	+	G	G	G	−	−	G	−	−	−	G	−
n-valeric	−	−	−	−	−	−	−	−	−	−	−	−
lactic	+	G	+	G	−	−	G	−	−	−	G	−
succinic	++	+	++	++	++	++	++	+	++	++	++	++

† pale brown after incubation for 7 days.

TABLE 1

Results obtained with the 40 test strains

| | B. melaninogenicus ss. melaninogenicus | | |